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Inflammation and matrix degrading proteases in coronary artery disease

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Stockholm 2006

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Published and printed by Reproprint AB, Stockholm
REPROPRINT AB
Stockholm 2006
www.reproprint.se
Gävlegatan 12 B
100 31 Stockholm
ISBN 91-7140-727-8

”Målet är ingenting,
vägen är allt.
Målet är vägen.”

Robert Broberg

To my family

ABSTRACT

Coronary artery disease (CAD) is a major cause of morbidity and death in the world today. The underlying process, atherosclerosis, is caused by lipid accumulation and inflammatory processes within the intimal layer of the vessel wall. Stable CAD is characterized by gradually expanding atherosclerotic plaques that narrow the lumen and, by restricting the blood flow, give rise to ischemic symptoms. Acute coronary syndromes (ACS) are usually caused by vulnerable plaques, which rupture or erode, thus initiating thrombus formation that suddenly impairs the blood flow and may cause acute myocardial infarction (MI).

Inflammation is now considered to be an important feature of both atherosclerosis and atherothrombotic complications. Inflammatory cells, such as macrophages, T-cells and mast cells, are present in the atherosclerotic tissue and interact with the cells present in the vascular wall.

Matrix metalloproteinases (MMPs), a family of more than 20 zinc- and calcium-dependent proteases, are capable of degrading extracellular matrix components. MMPs are involved in both the expansion of atherosclerotic plaques and the weakening of the fibrous cap, thus contributing to the development of vulnerable plaques. Inflammatory cells are suggested to be the major source of MMPs.

The aim of this thesis was to evaluate the roles of MMP-3 and MMP-1 in CAD and MI, and how the expression of these MMPs may be modulated by inflammation. This was addressed by genetic analyses of known and novel polymorphisms in the MMP-3 and MMP-1 genes, biochemical analyses of circulating MMP-3, MMP-1, and various cytokines, and angiographic analyses of the extent and severity of CAD. The individual studies were conducted in three different cohorts: i) the Stockholm Coronary Artery Risk Factor (SCARF) study, a case-control study of 387 postinfarction patients and 387 healthy control persons, ii) 164 patients from the Thrombolysis and REOCclusion (TREOC) study, a longitudinal cohort study of patients with ST-elevation MI, and iii) 1177 patients from the Southampton Atherosclerosis Study (SAS) with significant CAD as assessed by coronary angiography.

Serum MMP-3 concentrations were lower in postinfarction patients compared with control subjects. Also, serum concentrations of MMP-3 were lower in the acute stage of MI (within 48 hours) than at the three months follow-up visit. Plasma MMP-1 concentrations did not differ between postinfarction patients and controls.

The MMP-3 -1612 5A/6A promoter polymorphism markedly influenced the serum MMP-3 concentrations in two different cohorts of postinfarction patients and in healthy controls. Serum MMP-3 concentrations increased with the number of 6A-alleles. Furthermore, the serum MMP-3 concentration increased with the number of diseased major coronary arteries, as evaluated by coronary angiography.

Women had substantially lower serum MMP-3 concentrations compared with men in the SCARF and the TREOC study cohorts. In addition, plasma MMP-1 concentrations were lower in women in the SCARF study.

Studies of the MMP-1 gene revealed a haplotype effect of the MMP-1 –519 A/G and –340 C/T promoter polymorphisms on risk of MI. Compared with the A_{-519}-T_{-340} haplotype, both the A_{-519}-C_{-340} and G_{-519}-T_{-340} haplotypes showed a protective effect against MI whereas the G_{-519}-C_{-340} haplotype was associated with an increased risk of MI. Functional studies of gene expression demonstrated a lower promoter activity of the A_{-519}-C_{-340} and G_{-519}-T_{-340} haplotypes than of the A_{-519}-T_{-340} haplotype. Also, mRNA levels in atherosclerotic plaques were significantly lower in plaques retrieved from carriers of the A_{-519}-C_{-340} and G_{-519}-T_{-340} haplotypes than from carriers of the A_{-519}-T_{-340} haplotype.

Plasma concentrations of interleukin-2 (IL-2), IL-6, IL-8, and tumour necrosis factor-α were higher in postinfarction patients than in controls. Furthermore, there was a difference between MMP-3 and MMP-1 concentrations as regards their respective correlations with several circulating inflammatory cytokines. Correlations were found for MMP-1, but not for MMP-3.

In conclusion, the results presented in this thesis reinforce the novel alternative hypothesis of divergent effects of different MMPs in atherosclerosis and CAD. Inflammation is suggested to exert a stronger influence on MMP-1 than on MMP-3. Reduced MMP-3 concentration seems to be associated with MI, whereas increased concentration may promote progression of stable CAD.

Keywords: Myocardial infarction, matrix metalloproteinases, inflammation, polymorphism, haplotype, cytokines, gender

LIST OF ORIGINAL ARTICLES

This theses is based on the following original articles.

I

Samnegård A, Silveira A, Lundman P, Boquist S, Odeberg J, Hulthe J, McPheat W, Tornvall P, Bergstrand L, Ericsson CG, Hamsten A, Eriksson P.
Serum matrix metalloproteinase-3 concentration is influenced by MMP-3 -1612 5A/6A promoter genotype and associated with myocardial infarction.
J Intern Med 2005; 258: 411-9.

II

Samnegård A, Silveira A, Tornvall P, Hamsten A, Ericsson CG, Eriksson P.
Lower serum concentration of matrix metalloproteinase-3 in the acute stage of myocardial infarction.
J Intern Med 2006; 259: 530-6.

III

Pearce E, Tregouet DA, **Samnegård A**, Morgan AR, Cox C, Hamsten A, Eriksson P, Ye S.
Haplotype effect of the matrix metalloproteinase-1 gene on risk of myocardial infarction.
Circ Res 2005; 97: 1070-6.

IV

Samnegård A, Hulthe J, Silveira A, Ericsson CG, Hamsten A, Eriksson P.
Matrix metalloproteinase-1 (MMP-1) but not MMP-3 concentration is associated with levels of circulating inflammatory cytokines.
Manuscript

ABBREVIATIONS

AAA	Abdominal aortic aneurysm
ACS	Acute coronary syndrome
AIC	Akaike's information criterion
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease
CRP	C-reactive protein
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic mobility-shift assays
HDL	High-density lipoprotein
HRT	Hormone replacement therapy
IFN-γ	Interferon-γ
IL	Interleukin
IMT	Intima-media thickness
IVUS	Intravascular ultrasound
LD	Linkage disequilibrium
LDL	Low-density lipoprotein
MCP-1	Monocyte chemoattractant protein-1
MI	Myocardial infarction
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MT-MMP	Membrane-type MMP
PCI	Percutaneous coronary intervention
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
QCA	Quantitative coronary angiography
RFLP	Restriction fragment length polymorphism
SAS	The Southampton Atherosclerosis Study
SCARF	The Stockholm Coronary Artery Risk Factor study
SMC	Smooth muscle cell
SNP	Single nucleotide polymorphism
STEMI	ST-elevation myocardial infarction
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitor of metalloproteinases
TNF-α	Tumour necrosis factor-α
TREOC	The Thrombolysis and REOCclusion study

CONTENTS

INTRODUCTION

Cardiovascular disease (CVD) is the major cause of morbidity and death in the world today. One third of all deaths worldwide are due to different manifestations of CVD, mainly ischemic heart disease, stroke and peripheral artery disease[1]. The pathophysiology of atherosclerosis and arterial thrombosis is the common denominator of most aspects of CVD.

The arterial vessel wall

The unaffected human artery consists of three different layers[2,3]. Innermost is the intima with a monolayer of endothelial cells protecting the subendothelial proteoglycan layer from the blood stream, thus providing a barrier preventing prothrombotic molecules within the intima from coming into contact with platelets and coagulation factors in the blood. The outer boundary of the intima is the internal elastic lamina. The intima contains widely spaced single smooth muscle cells (SMCs) of both synthetic and contractile phenotypes. Synthetic SMCs are capable of synthesizing matrix components, including collagen, and of removal of lipoproteins. Contractile SMCs constitute the ”classical” phenotype, rich in myofilament and responding to mediators of the vascular tone. The matrix of the intima consists of collagens, proteoglycans, elastin, fibronectin and laminin.

The media, delimited by the internal and external elastic laminae, is inhabited mainly by contractile SMCs in spiralling layers, forming the muscular wall of the artery. The outermost layer, the adventitia, contains collagen fibrils, elastic fibres, fibroblasts and also some SMCs. The adventitia is providing nutrition to the vessel wall via the vasa vasorum, as well as the innervation of the artery.

Atherosclerosis

The pathological process of atherosclerosis takes place within the intima and involves modified lipids, inflammatory cells, SMCs and endothelial cells. Decades ago, atherosclerosis was considered a degenerative process caused by lipid overloading[2]. In the past twenty-five years, evidence has accumulated that inflammatory processes are crucial for initiation and progression of atherosclerosis, rupture of the fibrous cap and thrombotic complications leading to ischemia of the end organ, i.e. myocardial infarction (MI) in the case of coronary atherosclerosis[4,5].

Initiation and progression of lesions

In the initiation of atherosclerotic lesion formation lipoproteins infiltrate and are retained in the intima of the vessel wall[4,5]. Low density lipoproteins (LDLs) are then subjected to oxidative modification, which render them capable of activating endothelial cells to express adhesion molecules, most importantly vascular cell adhesion molecule-1 (VCAM-1). The adhesion molecules attract inflammatory cells, mainly monocytes and T-lymphocytes, from the blood stream and make them rolling over the endothelial surface. Eventually they migrate into the intimal layer in response to chemokines. These are specific for each cell type, e.g. monocyte chemoattractant protein-1 (MCP-1) for monocytes but also for T- and B-cells. Inside the vessel wall, monocytes transform into macrophages and phagocyte the modified LDL particles. Cholesterol accumulates and the macrophages evolve into foam cells. At this stage the lesions form so-called ”fatty streaks” which are present in human arteries already at an early age[6]. Later on, SMCs migrate into the intima and proliferate, thus constituting a substantial portion of the fibrous cap and producing ECM constituents. The activated macrophages secrete pro-inflammatory cytokines as well as matrix metalloproteinases (MMPs) and tissue factor, all substances that promote progression of atherosclerosis into advanced lesions, plaque rupture and thrombus formation[4,5]

The vulnerable plaque

The concept of the vulnerable plaque, a plaque prone to rupture, has been established since almost twenty years. The vulnerable plaque is characterized by a large, lipid-rich core, surrounded by clusters of macrophages and other inflammatory cells, and an overlying thin fibrous cap, with a reduced number of SMCs and a decreased amount of collagen[7-9]. The combination of an acellular lipid core, accumulation of macrophages in the shoulder region and a thin, collagen-poor fibrous cap makes the plaque less resistant to the mechanical forces of the blood stream. The fibrous cap can no longer resist the forces of the flowing blood and eventually it will break. As the fibrous cap ruptures, the extremely thrombogenic lipid-rich core is exposed to the blood stream, attracting platelets and coagulation factors to the site and thus initiating thrombus formation[8].

Inflammatory cytokines in atherosclerosis

The inflammatory cytokines are important mediators of the progression of atherosclerotic lesions. The vulnerable plaque is rich in inflammatory cells, such as T-lymphocytes producing the pro-inflammatory cytokines tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and CD40 ligand, macrophages and foam cells producing interleukin-1 (IL-1), IL-6, IL-8, IL-18, TNF-α, INF-γ, tissue factor and MMPs[5,10]. All these cytokines amplify the inflammatory cascade. In addition, neutrophils and mast cells are present in the vulnerable plaque. Neutrophils secrete neutrophil elastase and neutrophil collagenase (MMP-8) and mast cells produce serine proteases capable of activating MMPs[5,11].

T-cells differentiate further into T-helper1- (Th1-), Th2- or regulatory T-cells, the Th1-cells being the dominating T-cell type in atherosclerosis[4]. Th1-cells are the main source of IFN-γ and TNF-α. IFN-γ activates macrophages and endothelial cells, and decreases SMC and endothelial cell proliferation, SMC differentiation and SMC collagen production, thus exerting a pro-atherogenic effect[4]. In addition to Th1-cells, IFN-γ can be secreted by macrophages and cells of the vascular wall under the stimulation of other cytokines[12]. TNF-α, also a pro-atherogenic cytokine, is produced by Th1-cells and macrophages.

In a study of procollagen I and III gene expression in cultured vascular SMCs, transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) were shown to increase messenger ribo-nucleic acid (mRNA) expression whereas IFN-γ decreased mRNA expression and also wiped out the increasing effect of TGF-β and PDGF when the cells were co-cultured with IFN-γ, TGF-β and PDGF, respectively[13].

IL-1, produced by macrophages and SMCs, may contribute to recruitment and activation of leukocytes and platelets, regulation of cell growth, proliferation and migration of SMCs[14,15].

IL-6 is a potent cytokine produced by a wide range of cells including vascular and inflammatory cells. IL-6 stimulates macrophage activity and proliferation of SMCs, and induces acute phase protein production in the liver[16]. Also, IL-6 regulates the expression of IL-1β and TNF-α by endothelial cells.

IL-8 has more of chemokine properties, as it can attract leukocytes and enhances the adhesion of monocytes to the endothelium. In addition, it can promote angiogenesis in atherosclerotic tissue[10].

Cytokines produced by Th2-cells include IL-10 and IL-4. IL-4 stimulates antibody production and activates B-cells and mast cells in atherosclerosis, and may be considered pro-atherogenic[4]. Regulatory T-cells, together with Th2-cells and macrophages, are the source of the anti-inflammatory cytokines IL-10 and TGF-β. IL-10 inhibits T-cell and macrophage production of other cytokines, including INF-γ, and appears to have atheroprotective properties as IL-10 deficient mice develop larger atherosclerotic lesions than wild type mice, while transgenic mice overexpressing IL-10 show smaller atherosclerotic lesions[17]. TGF-β is thought to be atheroprotective through T-cell inhibition[4].

Circulating levels of cytokines

Several studies assessing the risk and/or severity of coronary artery disease (CAD) associated with different inflammatory markers have almost consistently shown modestly elevated levels of C-reactive protein (CRP), IL-1, IL-2 and IL-6 to be harmful, whereas the studies of TNF-α are fewer and their results more divergent[18].

Serum concentrations of TNF-α and IL-10 were higher in CAD patients than in controls, with no difference between stable and unstable CAD, whereas IL-2 was increased in unstable patients only as compared to controls[19]. In another study with a similar design plasma concentrations of TNF-α but not of IL-10 were elevated in CAD patients as compared to controls[20]. A third similar case-control study failed to detect differences between the groups in TNF-α levels whereas the IL-6 concentration was increased in CAD patients compared with controls[21].

Plasma levels of IL-8 were higher in men with unstable CAD than in men with stable CAD or healthy controls[22]. In an Italian study of stable and unstable CAD, the concentrations of IL-2 were elevated in both CAD groups whereas IL-6 concentration was elevated only in the unstable CAD group as compared with the control group[23]. In contrast, plasma IL-10 concentrations did not differ among the different groups. These diverging results were all except one[21] obtained in small studies with less than 120 participants.

The studies on IL-6 are more comprehensive and uniform. Increased IL-6 levels have been shown to predict future cardiovascular events in healthy individuals of both genders[24-26]. The plasma concentration of IL-6 at admission for MI was shown to be an independent prognostic factor, predicting death or new MI, after ST-elevation MI (STEMI)[27], whereas the CRP level was not[28]. Evaluating circulating IL-6 concentrations has been proposed for screening patients at risk of acute coronary syndrome (ACS)[16].

Coronary artery disease

CAD is the leading cause of death worldwide. In 2002, 12.6% of all deaths were caused by ischemic heart disease, the consequence of CAD. In Sweden, 30% of male and 23% of female deaths were due to CAD in year 2002, but numbers are decreasing possibly as a consequence of smoking cessation and lower blood pressure and cholesterol levels in the community (www.socialstyrelsen.se, Folkhälsorapport 2005). One year mortality after MI in Sweden in 2003 was 5% for patients below 60 years of age, 9.9% between 60-70 years, 21% for those aged 70-80 and approximately 35% over the age of 80[29].

Well-established risk factors for CAD include age, male gender, smoking, hypercholesterolemia, diabetes and hypertension. These risk factors do not explain all cases of MI, and additional factors may play a role in the complex interaction between genetic predisposition and environmental influences.

Stable angina pectoris

In stable angina pectoris, atherosclerotic plaques are slowly growing inwards, gradually impairing the lumen and blood flow. The patient experiences chest pain during exercise due to myocardial ischemia when the oxygen demand exceeds the oxygen supply. The chest pain usually subsides at rest, and returns with new exertion. Treatment aims at relieving symptoms and preventing MI, which can be achieved by three different approaches: i) by medications which reduce the oxygen demand or enhance blood flow, e.g. betablocking agents and vasodilators, or reduce the progression of atherosclerosis, e.g. lipid lowering compounds, ii) by percutaneous coronary intervention (PCI) aiming at restoring the blood flow by mechanically reducing the lumen restrictions caused by the plaques, and iii) by coronary artery bypass grafting (CABG), an open heart surgery procedure where the stenosed coronary arteries are by-passed using arterial or saphenous vein grafts[2].

Acute coronary syndrome

The pathophysiologic event precipitating ACS, i.e. unstable angina and MI, is thought to be thrombosis superimposed on the atherosclerotic plaque. ACS not infrequently seems to come out of a clear sky, as the atherosclerotic plaque may not impair blood flow as long as it not significantly decreases the lumen. However, when a thrombus occurs intraluminally it may cause impaired blood flow at rest or even total occlusion of the vessel leading to STEMI. Four different pathological entities have been recognized to cause ACS: i) plaque rupture is by far the most common and best defined cause of intraluminal thrombosis underlying nearly three quarters of all instances of fatal coronary thrombosis; ii) erosion of the endothelial layer exposing thrombogenic molecules; iii) erosion of calcium nodule; and iv) intraplaque haemorrhage arising from the microvasculature of the plaque itself[30].

Treatment for ACS is mainly aiming at reducing thrombus formation, thereby reducing the risk of propagation to total occlusion. In STEMI, where the affected artery is already occluded, restoration of blood flow by revascularization, PCI or thrombolysis, is the primary aim. In unstable angina and non-STEMI efforts are directed towards inhibition of thrombus propagation by blunting platelet activity and the coagulation cascade. This is achieved by aspirin, adenosine diphosphate-receptor antagonists and glucoprotein IIb/IIIa-inhibitors and with PCI and stenting of the culprit lesion[2].

Matrix metabolism in the fibrous cap

Synthesis and degradation of extracellular matrix (ECM) components are determinants of the fibrous cap composition and strength. SMCs are the main providers of collagen precursors, and the collagen synthesis is dependent on both the amount of SMCs and their synthesizing capacity. TGF-β and PDGF enhance the collagen synthesis in SMCs whereas IFN-γ markedly decreases this capacity[9]. IFN-γ may also inhibit SMC proliferation and promote SMC apoptosis[9]. Degradation of collagen and elastin is enhanced by different proteases released from most of the vascular cells, but most profoundly from macrophages. TNF-α, IL-1 and MCP-1 are cytokines known to stimulate protease secretion from vascular cells[9,10].

Proteases are enzymes capable of degrading proteins into amino acids by cleavage of peptide bonds. The proteases are subgrouped according to the active residue of the catalytic site of the enzyme into serine proteases, cysteine proteases, aspartic proteases and metalloproteinases. The metalloproteinases are further divided into metallocarboxipeptidases and metalloendopeptidases. The prefix "metallo-" indicates that the protease is dependent on a metal ion, i.e. Zn^{2+}, at its active site.

Matrix metalloproteinases (MMPs)

The matrix metalloproteinases (MMPs), a family of zinc- and calciumdependent proteolytic enzymes, are members of the metalloendopeptidases, and are capable of degrading ECM components of various tissues, thus being implicated in the degradation of the fibrous cap leading to plaque rupture. The MMPs are involved in a number of physiological processes encompassing matrix degradation, such as embryonic development, wound healing, tissue reabsorption and inflammation[31]. The action of different MMPs is implicated in cell migration, reorganization of tissues, regenerative processes, neovascularization and tumour spreading[31]. Generally MMPs are grouped into five different classes according to their structure and substrate specificity; these are collagenases, gelatinases, stromelysins, membrane-type MMPs (MT-MMPs) and others (Table 1). In all, there are more than 20 different MMPs identified in humans[32]. The amino acid sequence of different MMPs shows about 40% homology. The principal structure of MMPs is composed of three domains; pro-peptide, catalytic domain and hemopexin-like domain. MT-MMPs have an additional domain, the transmembrane domain.

Regulation of MMP activity

The consequence of MMP activity, i.e. degradation of structural and matrix molecules, could be deleterious if it would appear at inappropriate sites and time points. To prevent such hazardous incidents the activity of the MMPs is tightly regulated. This regulation takes place at three different levels; altered gene transcription, extracellular activation of pro-enzymes and inhibition of activated MMPs by tissue inhibitors of metalloproteinases (TIMPs). The expression of MMP genes is known to be influenced by cytokines, growth factors and hormones. For example IL-1 and TNF-α upregulate transcription, whereas TGF-β and corticosteroids inhibit MMP expression[33, 34]. Activation of the pro-enzymes takes place extracellularly, and the main activator of MMPs is plasmin, even though there is some autoactivation by the MMPs themselves as well as activation by other proteases like the serine proteases. The third regulatory level is the inactivation of MMPs by TIMPs. There are four different TIMPs, TIMP-1 and TIMP-2 being the most studied forms. They act by an irreversible binding to the MMP and block the catalytic site[33]. TIMPs are often co-expressed with MMPs, probably to create a local and tightly regulated proteolytic activity.

Table 1: The major matrix metalloproteinases (MMPs) and their substrates

Group	MMP	Trivial name	Substrate
Collagenases	**MMP-1**	Interstitial collagenase	Collagens I, II, III, VII, VIII and X, MMP-2, MMP-9
	MMP-8	Neutrophil collagenase	Collagens I, II, III, V, VII, VIII and X, fibronectin
	MMP-13	Collagenase-3	Collagens I, II, III and V
Gelatinases	MMP-2	Gelatinase A	Gelatin, collagens I, IV, V, VII, X, XI and XIV, elastin, fibronectin, MMP-9, MMP-13
	MMP-9	Gelatinase B	Gelatin, collagens IV, V, VII, X and XIV, elastin, fibronectin
Stromelysins	**MMP-3**	Stromelysin-1	Collagens III, IV, IX and X, elastin, laminin, proteoglycans, fibronectin, MMP-1, MMP-7, MMP-8, MMP-9, MMP-13
	MMP-10	Stromelysin-2	Collagens III, IV and V, elastin, fibronectin, MMP-1, MMP-8
	MMP-11	Stromelysin-3	
Membrane type	MMP-14	MT1-MMP	Collagens I, II and III, fibronectin, laminin vitronectin, MMP-2, MMP-13
	MMP-15	MT2-MMP	Fibronectin, laminin, MMP-2
	MMP-16	MT3-MMP	MMP-2
	MMP-17	MT4-MMP	
Others	MMP-7	Matrilysin-1	Collagens IV and X
	MMP-12	Metalloelastase	Collagen IV, elastin, fibronectin, vitronectin, laminin

Adapted from Beaudeux et al. 2004

MMPs can be expressed by various cells, such as chondrocytes, myocytes, osteoblasts, and epithelial, endothelial and inflammatory cells[35]. In the vascular wall all major cell types can express MMPs in response to stimulation. Accordingly, MMP expression occurs in macrophages, SMCs, endothelial cells, T-lymphocytes, mast cells and fibroblasts[36]. In atherosclerosis, macrophages have been suggested to be the main source of MMP expression. In an experimental study of gene expression in THP-1 monocytes undergoing differentiation into macrophages, MMP-1, MMP-2, MMP-9 and MMP-14 were upregulated, whereas MMP-17 expression did not change, and other MMPs, including MMP-3, were not expressed either before or after differentiation[37]. This finding was confirmed in human primary monocytes and macrophages.

MMPs in atherosclerosis

In the normal arterial wall, only MMP-2 is constitutionally expressed along with TIMP-1 and TIMP-2[38]. In atherosclerosis, on the other hand, there is strong evidence for expression of several MMPs in plaques, including MMP-1, MMP-2, MMP-3, MMP-9 and MMP-13[38-41].

In recent years new technologies to study protein effects have emerged. Mouse models, using a knockout strategy (silencing of the gene) or a transgenic approach (overexpression of the gene), have proven to be valuable tools to understand the impact of different proteins on disease development[42]. Mouse models are convenient as the animals have a relatively short life span. As mice do not develop atherosclerosis spontaneously, genetically modified mice strains have been engineered to get atherosclerotic prone mice. One common model is the apoE knockout mouse in which advanced atherosclerotic lesions similar to those occurring in humans appear. ApoE knockout mice fed a Western type diet become extremely hypercholesterolemic and develop atherosclerosis even more rapidly. However, in general these mice do not develop plaque rupture followed by thrombotic complications. Another model of atherosclerosis is the LDL-receptor deficient mouse and a number of transgenic mice expressing human genes. Unfortunately there is not yet any optimal model to study spontaneous plaque rupture with ensuing thrombosis.

Several MMPs have been knocked out without conclusive findings. Importantly, it is a known

phenomenon when performing such experiments that there can be compensatory mechanisms accounted for by other related molecules. In a study by Johnson et al. the authors conclude that MMPs have divergent effects, suggesting MMP-3 and MMP-9 to be anti-atherogenic, MMP-12 to be pro-atherogenic and MMP-7 to be indifferent as regards atherogenesis[43].

In a study by Silence et al. the apoE/MMP-3 double knockout mouse was studied in relation to atherosclerosis[44]. The double knockout mice developed more severe atherosclerosis, as measured by percentage lumen area containing atherosclerotic plaques, than the single apoE knockout mice. The plaques of the double knockout mice contained less macrophages and had a lower lipid, but higher collagen content. In addition, aneurysms of thoracic and abdominal aortas were less frequent in the apoE/MMP-3 double knockout mice than in single apoE knockout mice. These results were to a large extent confirmed in the study by Johnson et al. investigating the brachiocephalic arteries of apoE deficient mouse strains in which one of the respective MMP-3, MMP-7, MMP-9 or MMP-12 genes had been knocked out[43]. In the apoE/MMP-3 double knockout mice the plaque area was greater, the number of buried fibrous layers per plaque (suggesting previous plaque rupture) was higher, and the content of SMC was lesser, whereas the content of macrophages did not differ in relation to the apoE single knockout mice. The same findings pertained to MMP-9 except that apoE/MMP-9 double knockout mice also had a higher content of macrophages in their plaques as compared to apoE single knockout mice. In apoE/MMP-7 knockouts there were no differences except for SMC content, which was higher in double knockouts as compared to apoE single knockouts. Finally, in the apoE/MMP-12 double knockouts the plaque area was smaller, the number of buried fibrous layers per plaque was lower, the SMC content higher and the macrophage content lower than in the apoE single knockout. Deguchi et al. have studied atherosclerosis in apoE/MMP-13 deficient mice on an atherogenic diet[45]. After 10 weeks the double knockout mice and the single apoE knockout mice had similar plaque area and plaque content of macrophages and SMCs whereas the double knockout mice had a higher collagen content in their plaques.

There are no reports on MMP-1 knockout mice in the literature. The predominant opinion was long that no MMP-1 existed in the mouse, as only MMP-13 was detected in mouse tissue. In 2001, Balbin et al. succeeded in cloning two new MMPs in the MMP gene cluster on mouse chromosome 9 and at least one of them, murine collagenase-like A (Mcol-A) could represent the orthologue of human MMP-1[46]. Mcol-A and Mcol-B were predominately present during mouse embryogenesis.

Transgenic mice expressing human MMP-1 in macrophages on a apoE deficient background and fed a Western diet for 16 weeks had earlier and less extensive atheromatous lesions with less collagen staining of the intima than apoE deficient mice lacking the human MMP-1 gene[47]. No difference was detected in SMC or macrophage content. There was no evidence of plaque rupture in the MMP-1 expressing mice.

MMP-3

MMP-3, also denoted stromelysin-1, is an MMP showing activity against a wide range of substrates, e.g. collagen types III, IV, IX and X, proteoglycans, laminin, elastin and fibronectin. MMP-3 is also involved in the activation of other MMPs (e.g. proMMP-1, proMMP-8, proMMP-9 and proMMP-13) as well as in autoactivation of pro-MMP-3[48]. Importantly, MMP-3 is not capable of degrading the structurally essential type I collagen fibres, which are highly responsible for the strength of the fibrous cap[8,9].

The effects of MMP-3 have been studied in numerous clinical settings. Elevated serum and/or plasma concentrations of MMP-3 have been associated with a wide range of clinical conditions, such as rheumatoid arthritis, systemic lupus erythematosis[49], rapidly destructive osteoarthritis of the hip[50], and different types of cancer, especially in the metastatic phase[51]. Decreased plasma concentrations of MMP-3, on the other hand, have been found to be associated with agnogenic myeloid metaplasia, implicating that a lack of MMP-3 may play a role in fibrosis of the bone marrow[52].

In the atherosclerotic vessel wall, MMP-3 is expressed by vascular SMCs, macrophages and endothelial cells. In addition, by immunoflour-

escense technique, MMP-3 was found to be present in ECM in areas of SMC proliferation in inflammatory bowel disease, both Crohn´s disease and ulcerative colitis, but not in normal tissue from the same patients[53]. This latter finding indicates that MMP-3 enhances SMC migration and proliferation.

MMP-3 in atherosclerosis

Evidence of the presence of MMP-3 in atherosclerotic plaques was first reported by Henney et al. in 1991[39], with use of *in situ* hybridisation technique applied on atherosclerotic plaques obtained from coronary arteries of heart transplant recipients. The MMP-3 mRNA signal co-localized both with SMCs and a subpopulation of macrophages containing visible intracellular lipid deposits[39]. A few years later, Galis et al. reported occurrence of MMP-3, along with MMP-1, MMP-2 and MMP-9, in human atherosclerotic tissue, specifically in SMCs and macrophage-derived foam cells. In addition, areas rich in lymphocytes also co-localized with MMP-3 and the other MMPs tested[38].

In a study by Schoenhagen et al. increased cell-associated MMP-3 staining was associated with positive arterial remodelling. The quantities of inflammatory and interstitial cells were equal in both positively and negatively remodelled vessels, thus suggesting that MMP-3 expression is not merely a consequence of increased inflammation[54].

Measuring the MMP-3 concentration in the circulation is a blunt but relatively easy way to evaluate a possible influence of MMP-3 in different conditions. A drawback of most assays of MMP-3 is that they are measuring both pro-MMP-3, activated MMP-3 and MMP-3 bound to and inhibited by TIMP. This makes it difficult to estimate MMP-3 activity, not least the MMP-3 in the vessel wall. In addition, very little is known about the clearance of MMP-3 from the circulation.

Studies of MMP-3 concentration in serum or plasma of patients with manifestations of CAD have reached divergent results. In a study by Noji et al., male patients with stable CAD had lower plasma MMP-3 concentration than healthy controls[55] whereas Inoue et al. found the serum MMP-3 concentration to be higher in coronary sinus blood in patients with ACS than in patients with stable CAD or in healthy control persons[56], even though there were no differences when measuring the concentration in arterial blood. Also, Yan et al. reported higher serum MMP-3 concentration in patients with ACS than in healthy controls or patients with stable CAD[57]. Furthermore, Wu et al. demonstrated that the MMP-3 level is higher in patients with stable angina pectoris than in controls and also that higher levels of MMP-3 predicted a worse outcome in patients with CAD[58]. In contrast, studies of both hyperlipidemic[59] and diabetic[60] patients showed that affected individuals had lower levels of MMP-3 than the corresponding controls. In a study of hyperlipidemic but otherwise healthy subjects, Beaudeux et al. found the serum levels of MMP-3 to be higher in subjects with carotid plaques than in subjects without signs of carotid plaques as estimated by carotid ultrasound examination[61]. Also, hyperlipidemic subjects presented higher MMP-3 concentrations than controls.

MMP-1

MMP-1, interstitial collagenase, is capable of degrading intact type I collagen fibres, which are otherwise resistant to most proteases, including MMP-3. Collagen is the main extracellular component of the fibrous cap of advanced atherosclerotic lesions. Most collagen is composed of type I collagen fibres, about 70% of the total collagen content[8,62]. The collagenases are required for the initial degradation of the tightly winded collagen triple helical structure. The resulting collagen remnants are then accessible to further degradation by other MMPs. Hence, MMP-1, along with MMP-8 and MMP-13, is implicated in the degradation of the fibrous cap.

MMP-1 in atherosclerosis

There is no evidence of MMP-1 expression in normal human arteries while it has been detected in human atherosclerotic lesions[38,41]. Also, in a study of carotid artery lesions, MMP-1 gene expression was considerably increased in plaques with a thin fibrous cap compared with plaques with a thick fibrous cap[63]. MMP-1 present in the atherosclerotic plaque is principally expressed by macrophages in the shoulder region and the regions adjacent to the lipid core, but also, to a lesser extent, by SMCs[38,41].

MMP-1 has been proposed, in conjunction with MMP-13, to increase collagenolysis in human atherosclerotic plaques[40]. Similarly, the inflammatory cytokines IL-1β, TNF-α and IFN-γ, possibly capable of inducing MMP expression, are expressed in the atherosclerotic lesions, but undetectable in normal arteries. *In vitro* experiments in THP-1 monocytes differentiated into macrophages showed a 6.6-fold increase in the expression of MMP-1 on differentiation, the increase being most pronounced shortly after stimulation[37].

There is limited information on circulating levels of MMP-1 in the setting of stable CAD. No difference in serum MMP-1 concentration was detected between patients with hemodynamically significant coronary stenosis and those without, in an angiography study[64]. In a subgroup analysis of the patients with significant CAD, those who had at least one complex lesion had higher levels of MMP-1 than those who had no complex lesion as defined by angiographic criteria[64]. Also, two studies on stable CAD have failed to detect any MMP-1 in circulating blood[65,66]. In a study of abdominal aortic aneurysm (AAA), no difference in plasma concentrations of MMP-1 was seen between patients with AAA, patients with obliterative aortic atherosclerosis and healthy controls[67].

In a study by Inoue et al., the serum concentration of MMP-1 in coronary sinus blood was higher in patients suffering ACS than in patients with stable angina pectoris or in healthy individuals undergoing coronary angiography because of chest pain whose angiograms were normal[56]. In contrast, Fukuda et al. did not detect any difference in serum concentration of MMP-1 between patients with acute MI, unstable angina pectoris or stable CAD, nor was there any difference between patients with and without signs of plaque rupture on investigation with intracoronary ultrasound[68].

Six studies have investigated MMP-1 levels at different time points during the acute stage of MI and the first month of recovery[69-74]. The earliest one, presented in 1997, had no control group included, but could detect an increase in serum concentration of MMP-1 at one and two weeks after the index event[69]. The remaining five studies included a control group of either healthy individuals[70,72,74] or patients with CAD but without signs of acute MI[71,73]. There was no significant difference in the levels of MMP-1 between MI patients and controls at any time point investigated in two of these studies[70,74]. In two other studies, the levels of MMP-1 were higher in the MI group already at admission, as compared with the control group[71,72], and in one of these, the levels increased significantly at one and two weeks after the MI[71]. On the other hand, Eckart et al. did not detect any difference in serum MMP-1 level at admission between patients with and without MI, but there was a significant increase in MMP-1 levels in MI patients 24 hours later[73]. Of note, only one study demonstrated elevated levels in patients at one month after the index MI as compared with controls[71]. Overall, these studies show fluctuating MMP-1 levels in the course of MI, as levels increase either early (1-2 weeks) or very early (24 hours) after the onset of MI, and then diminish early as they have returned to baseline at one month. Accordingly, MMP-1 might be involved in the acute inflammatory process of MI.

Genetic regulation of MMP expression

Deoxyribonucleic acid (DNA) with the four nucleotides adenine (A), cytosine (C), guanine (G), and thymine (T) is the foundation of all living creatures and plants, essentially storing the complete information needed for all biological processes such as embryogenesis, development and daily life. The sequence of nucleotides decides the amino acid sequence of the proteins and thereby their structure and function

A defined sequence of DNA constitutes a gene. There are approximately 32.000 genes in the human genome[42,75]. Most genes include a promoter region and a transcription unit with exons and introns. Protein synthesis starts with the transcription of DNA into mRNA, a molecule transferring the genetic information from DNA to protein synthesis. The transcription starts with formation of the transcription complex, including RNA polymerase II and several transcription factors attaching to the promoter region of the gene. The nucleotide sequence of the promoter region of the gene influences the binding properties of transcription factors. Transcription factors are nuclear proteins acting as either activators or repressors of the transcriptional activity, in this way modulating

protein synthesis. During splicing, mRNA is modified as the introns are deleted and then translated into an amino acid sequence, where a nucleotide triplet corresponds to one amino acid. Eventually the amino acid sequence folds into the protein structure intended.

DNA polymorphisms are naturally occurring variations in the nucleotide sequence that may influence the phenotype. Four types of polymorphisms are identified: i) single nucleotide polymorphisms (SNPs), "misspellings" where a single nucleotide has been switched, e.g. G instead of A, ii) insertion of a nucleotide, e.g. 6A instead of 5A, iii) deletion of a nucleotide, i.e. one nucleotide is missing, and iv) microsatellite repeats, short nucleotide sequences that are repeated a significant number of times. There are currently more than 10 million SNPs listed in the National Institutes of Health (NIH) database of SNPs (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi), including insertion/deletion and small microsatellite repeats. These polymorphisms may, depending on their location, influence expression of the gene or function of the protein product. Examples on polymorphisms influencing transcription, splicing and mRNA stability have been described.

Haplotype, or more specifically "haploid genotype", is a specific combination of alleles within one single gene or in related genes on the same chromosome. Alleles included could be more or less linked to each other. Single polymorphisms of a gene may not influence the phenotype but when combining into haplotypes they might. The closer two alleles are situated on the chromosome, the more linked they are, and the more likely they will be passed on to the next generation together. Thus, the haplotype reflects the linkage disequilibrium (LD), which can be estimated by calculating the so-called LD coefficient. The genes coding for several MMPs (MMP-1, MMP-3, MMP-7, MMP-8, MMP-10, MMP-12, MMP-13, MMP-20 and MMP-27) are located in a cluster in the same region on chromosome 11 implicating that the alleles of these MMPs may be in strong LD[33] (www.ensemble.org).

Atherosclerosis and CAD are multifactorial diseases. The set of alleles of the individual interacts with environmental modifying factors to influence the risk of the individual to attract CAD. The association of a genotype with the studied disease outcome, i.e. MI or CAD, is virtually a statistical phenomenon that hardly translates into predicting the risk of a single individual as the predisposing genetic risk is greatly modified by environmental agents that change over time and interact to influence genetic susceptibility. Association studies can be performed either with a genome-wide screening approach, which is unbiased in relation to assumptions on particular genes, or with a candidate gene approach. The latter approach addresses an a priori appointed gene, which could be of interest e.g. from functional studies. Hundreds of genes, each with various variants, have been suggested to influence the risk of MI and CAD in association studies[76] but the newly presented INTERHEART study of 15.152 MI patients and 14.820 controls investigated in 52 countries revealed nine, mainly environmental, risk factors accounting for 90% of the risk in men and 94% of the risk in women to suffer MI[77]. This study reinforces the notion that atherosclerosis and its clinical manifestations are very complex conditions where we will not find single gene polymorphisms to have more than quite minor effects.

MMP-3 -1612 5A/6A – a functional polymorphism

The gene coding for MMP-3 is located on the long arm of chromosome 11 in the region 11q22.2-22.3[78]. In 1995, Ye et al. identified a common polymorphism in the promoter region of the MMP-3 gene, located 1612 base pairs upstream of the transcription start site. This polymorphism is an insertion/deletion polymorphism with one allele containing a run of five adenosines (5A), and the other allele containing a run of six adenosines (6A)[79]. The earlier designation of this polymorphism, which still appears in the literature, has been -1171 5A/6A as it was initially sequenced from a clone with an inversion. Beyzade et al. identified an additional six SNPs in the MMP-3 gene in 2003, but the effect of MMP-3 gene variation was mainly attributable to the -1612 5A/6A promoter polymorphism[80].

Transient transfection experiments in cultured fibroblasts and vascular SMCs have indicated a

functional role of the polymorphism since the 5A-allele expressed a two-fold higher activity of the reporter gene than did the 6A-allele[48]. The allele-specific expression is probably caused by a repressor nuclear protein binding preferentially to the 6A-allele and resulting in lower expression of MMP-3. This hypothesis has been supported by the finding that the MMP-3 gene expression in skin biopsies of healthy men was significantly lower in individuals homozygous for the 6A-allele compared with those homozygous for the 5A-allele, as was MMP-3 protein expression[81].

The allele frequency of the MMP-3 -1612 5A/6A polymorphism appears to be quite different in a Caucasian compared with an Asian or Afro-American population. Whereas the 5A-allele frequency in the Caucasian population is about 50%, it is less than 15% in an Asian or Afro-American population[82].

MMP-3 -1612 5A/6A polymorphism and circulating MMP-3

The first study to show a clear association of circulating levels of MMP-3 to the MMP-3 -1612 5A/6A promoter polymorphism was the one of Mattey et al., exploring 197 patients with the diagnosis of rheumatoid arthritis. Among these patients the serum MMP-3 concentration was strongly associated with the 5A/6A polymorphism as the serum concentration increased with the number of 6A-alleles[83]. The same result has later been reached in an Australian cohort of CAD patients, as reported in an abstract presented by White et al. at the American Heart Association Scientific Sessions of 2005[84]. The findings of increasing levels of MMP-3 with a rising number of 6A-alleles are intriguing, as earlier in vitro studies have reported the 5A-allele to increase the expression of MMP-3[48].

Two smaller studies, both exploring carotid intima-media thickening (IMT), have failed to observe any relationship of circulating levels of MMP-3 to the 5A/6A genotype[85,86]. In yet another study, 103 patients with rheumatoid arthritis the serum MMP-3 concentration did not differ between the genotypes[87].

MMP-3 -1612 5A/6A polymorphism and risk of CAD

The MMP-3 gene harbouring the -1612 5A/6A promoter polymorphism was early recognized as a candidate gene for several conditions, including atherosclerosis. Several reports are found in the literature on the association of the MMP-3 -1612 5A/6A polymorphism and the risk of MI and severity of CAD. Regrettable, these studies have reached divergent results (Table 2 and 3) and it is difficult to arrive at a comprehensive conclusion as they have been performed in various populations, differ in gender mix and have studied different outcomes of CAD. When it comes to extension and severity of CAD, as measured by coronary angiography, most of the studies have shown the 6A-allele to be associated with occurrence or more severe disease[80,84,88,89] as well as with faster CAD progression[79,90-92] (Table 2). However, one of them, notably the largest one, failed to detect any genotype-specific differences[93]. Only one study indicated the 5A-allele to confer higher risk of angiografically proven CAD[94]. In addition, in an autopsy study, Pöllänen et al. found the 5A-allele to be associated with larger calcified lesions in the coronary artery trees of male sudden death victims, 53-69 years old, in Finland[95] (Table 4).

In the association studies of risk of MI, summarized in Table 3, the majority of the studies show the 5A-allele to be associated with higher risk[80,96-99] whereas one study points to the 6A-allele as being harmful[100]. In addition, two studies failed to prove association with either allele[88,95].

Four studies have explored a divergent mix of CAD outcomes (Table 4). Two of them studied the risk in healthy individuals[101,102] and the other two studied the risk of a recurrent event in patients who already had suffered a manifestation of CAD[91,103]. In the study by Humphries et al.[101], healthy men carrying the 6A-allele had a higher risk of an event (MI, CABG or death) than 5A-allele carriers. In contrast, Ye et al. could not demonstrate any association of the polymorphism with the combined endpoint of MI, angina pectoris, CABG or PCI[102]. In the two studies of prognosis after a CAD event, the first one confirmed the 6A-allele to be harmful as patients carrying the 6A-allele had twice as many events (MI, CAD death, symptom driven intervention, stroke or transient ischemic attack (TIA)

Table 2. Association studies of MMP-3 -1612 5A/6A promoter polymorphism on risk/severity/progression of angiographic coronary artery disease

Reference	n, total or P/C	Population	% male, total or P/C	6A-allele freq, total or P/C	Higher risk allele
Schwarz et al.[88]	1848	Caucasian	100	0.49/0.51	6A
Hirashiki et al.[89]	1011/650	Asian	69/56	N.A.	6A
Beyzade et al.[80]	1070	Caucasian	67	0.49	6A
White et al.[84]	44*	Caucasian	N.A.	N.A.	6A
Ye et al.[79]	72	Caucasian	100	0.51	6A
Humphries et al.[90]	187**	Caucasian	100	0.60	6A
deMaat et al.[91]	249**	Caucasian	100	0.49	6A
Humphries et al.[92]	287 ***	Caucasian	85	0.52	6A
Hoppman et al.[93]	2667	Caucasian	75	0.51	No difference
Kim et al.[94]	131/117	Asian	68/50	0.76/0.85	5A
Paper I	243	Caucasian	82	0.53	No difference

P/C Patients/Controls; *plaque area as estimated by intravascular ultrasound (IVUS); **placebo treated group only; ***balloon treated group only; N.A., not available

Table 3: Association studies of MMP-3 -1612 5A/6A promoter polymorphism and risk of myocardial infarction

Reference	n, total or P/C	Population	% male, total or P/C	6A-allele freq, total or P/C	Higher risk allele
Terashima et al.[96]	330/330	Asian	82/78	0.70/0.82	5A
Beyzade et al.[80]	132/780	Caucasian	67	0.43/0.50	5A
Nojiri et al.[97]	466/335	Asian	86/63	0.82/0.88	5A
Liu et al.[98]	150/150	Asian	84	0.65/0.80	5A
Zhou et al.[99]	509/518	Asian	82/78	0.81/0.86	5A
Yamada et al.[100]	2374/1778	Asian	75/60	0.85/0.79	6A
Schwarz et al.[88]	1848/515	Caucasian	100	0.49/0.51	No difference
Pöllanen et al.[95]	86/204	Caucasian	100	0.59	No difference
Paper I	374/385	Caucasian	82	0.53/0.49	No difference

P/C Patients/Controls

and death of all causes) as patients homozygous for the 5A-allele during 2 years of follow-up[91]. This study, including patients with stable angina and at least one significant coronary stenosis was a secondary preventive, placebo-controlled study of pravastatin, a lipid-lowering drug. Association between the 6A-allele and events was only present in the placebo group. The second study reported on 5A/6A polymorphism in relation to prognosis after a first MI. The 5A/6A polymorphism was recognized as an independent predictor of a new event (recurrent angina, non-fatal MI or cardiac death) as the frequency of the 5A-allele was higher in the event group, resulting in an adjusted hazard ratio of 2.51 for patients carrying the 5A-allele[103].

Summarizing all studies concerning the association of the MMP-3 -1612 5A/6A promoter polymorphism with different manifestations of CAD, there is a discernible pattern of the 6A-allele promoting atherosclerosis progression in a stable manner whereas the 5A-allele seems to increase the risk of acute complications of atherosclerosis like MI, thus implicating a higher risk of developing vulnerable plaques. This line of reasoning is supported by studies of atherosclerosis in other arterial localizations (Table 4). Two studies on carotid IMT[85,104] and one of carotid stenosis[105] found the 6A-allele to be associated with more advanced lesions, while the 5A-allele has been associated with ischemic stroke, the acute complication of atherosclerosis of the carotid artery[106]. In contrast, the largest study in this field, comprising 1109 healthy individuals, could not detect any association of the 5A/6A polymorphism with carotid IMT[107]. In yet another study, both the 5A/5A and the 6A/6A genotypes had greater carotid IMT than heterozygotes[86]. Finally, a study of aortic stiffening in healthy subjects older than 60 years also showed both homozygous groups to have higher large artery stiffness than the heterozygous group[81].

Table 4:
Association studies of MMP-3 -1612 5A/6A promoter polymorphism in different manifestations of atherosclerosis

Reference	Condition or outcome studied	n, total or P/C	Population	% male, total or P/C	6A-allele freq, total or P/C	Higher risk allele
Pöllänen et al.[95]	Calcified lesion on autopsy, age >=53	145	Caucasian	100	0.60	5A
Humphries et al.[101]	Risk of MI + CABG + death	125/348	Caucasian	100	0.55/0.45	6A
Ye et al.[102]	Risk of MI + angina + CABG + PCI	471	Caucasian	58	0.46/0.49	No difference
de Maat et al.[91]	New event of MI + CAD death + CABG + PCI + stroke + TIA + death of all causes	249	Caucasian	100	0.49	6A
Liu et al.[103]	New event of MI + angina + cardiac death	49/121	Asian	84	0.58/0.72	5A
Gnasso et al.[85]	Carotid IMT	40	Caucasian	100	0.52	6A
Rundek et al.[104]	Carotid IMT	87	Caucasian	45	0.69	6A
Ghilardi et al.[105]	Carotid stenosis	91/133	Caucasian	59/65	0.62/0.50	6A
Flex et al.[106]	Ischemic stroke	237/223	Caucasian	56/48	0.56/0.60	5A
Beilby et al.[107]	Carotid IMT	1109	Caucasian	50	0.51	No difference
Rauramaa et al.[86]	Carotid IMT	96	Caucasian	100	0.56	Both homozyg
Medley et al.[81]	Aortic stiffening, age >=60	55	Caucasian	68	0.56	Both homozyg
McGlinchey et al.[108]	Risk of MI + UAP + pos. cor. angiogram	368 families*	Caucasian	80, affect sib	0.45	No difference

P/C Patients/Controls; *combined transmission disequilibrium test (TDT)/sib-TDT and pedigree disequilibrium test (PDT); MI, myocardial infarction; CABG, coronary artery bypass grafting; PCI, percutaneous coronary intervention; CAD, coronary artery disease; TIA, transitory ischemic attack; IMT, intima-media thickness; UAP, unstable angina pectoris

In recent years new forms of genetic association studies have emerged. To refine the case-control approach, affected individuals are collected together with unaffected siblings and/or parents. In one study of family-based association, the combined transmission disequilibrium test (TDT)/sib-TDT and the pedigree disequilibrium test (PDT), failed to confirm any association of the 5A/6A polymorphism with CAD, as defined by a history of acute MI, unstable angina or significant CAD at angiography[108] (Table 4). In the latter study, the outcome tested combined the acute MI and the stable CAD phenotypes, which may have masked associations with the different phenotypes going in opposite directions. In addition, the number of studied families was quite small even though the post-hoc power calculations done by the authors stated that the study had >80% power to detect a deviation of allele transmission from 50% to 60% using the combined sibTDT/TDT.

MMP-1 polymorphisms

The gene coding for MMP-1 is located in the q21-22.1 region of chromosome 11[78]. One common polymorphism in the promoter region was identified already in 1998, the MMP-1 -1607 G/GG promoter polymorphism, and it has subsequently been extensively investigated in various diseases, predominantly different malignancies[109-111]. In an experimental study in fibroblasts and melanoma cells, the GG allele was found to be associated with higher transcriptional activity[109]. This MMP-1 promoter polymorphism has also been studied in relation to atherosclerosis by Ye et al., who reported lower risk of CAD in GG homozygotes than in G homozygotes[102]. In contrast, no association was found with the risk of MI in a larger study by Nojiri et al.[97]. In addition, no association was found between -1607 G/GG genotype and atherosclerotic lesion development in the abdominal aorta of young men in an autopsy study conducted by Nordskog et al.[112], nor was the MMP-1 -1607 G/GG promoter polymorphism found to be associated with carotid stenosis[105].

The MMP-1 -422 T/A, -340 T/C and -320 T/C promoter polymorphisms were identified by Thiry-Blaise et al. in 1995 in a study of osteoporosis, but did not influence the risk of disease when analysed individually[113], and no haplotype analysis was performed.

Jurajda et al. reported the presence of the MMP-1 -519 A/G promoter polymorphism in 2002, but no report is available suggesting that this polymorphism is associated with any disease[114]. Also, this polymorphism was closely linked to the aforementioned, as the A_{-519} allele more often occurs with the GG_{-1607} allele, and the G_{-519} allele more often occurs with the G_{-1607} allele[114].

Gender aspects of CAD

The incidence of MI in Sweden in the last ten years has been about four-fold higher among men than women younger than 60. At ages over 80, the incidence is similar. Conversely, in the age group below 60 years, the one-year mortality rate is higher among women (5%) than men (4%)[29]. Thus, women contract symptomatic CAD less frequently at younger age, but when they do, their prognosis is more dismal. As women become post-menopausal, the risk of CAD and MI gradually increases, and it has been proposed that oestrogen would exert a protecting effect[115-117]. However, hormone replacement therapy (HRT) with a combination of oestrogen and progestin has not proven to protect post-menopausal women from CVD, neither as a secondary preventive strategy as was tested in the Heart and Estrogen/progestin Replacement Study (HERS)[118], nor as primary prevention as illustrated by the early interruption of the oestrogen and progesterone treatment arm of the Women's Health Initiative (WHI) study[119]. In both these studies HRT tended to increase the CAD event rate. In a review published by the Cochrane Collaboration in 2005, one of the conclusions is that HRT is inappropriate for the prevention of chronic disease, including CAD[120].

In the past five years, 3850 patients (35% women) underwent coronary angiography at Danderyd Hospital according to the Swedish Coronary Angiography and Angioplasty Registry (SCAAR). The proportion of women having no significant lesion (>50% diameter stenosis) was 45% whereas the corresponding figure was 20% for men. This is consistent with less extensive CAD in women found in several other registry and interventional studies where gender differences have been explored[121-126].

Histological studies of coronary thrombosis in sudden death victims have revealed a higher fre-

quency of thrombus overlying a superficial erosion instead of plaque rupture in women than in men[127]. In addition, in the eroded plaques the infiltration of macrophages and T-cells was less pronounced, and clusters of SMCs more common than in the ruptured plaques, indicating a lower inflammatory activity. The plaque rupture etiology has been shown to be more frequent in women over 50 years of age than in women below 50 years[128]. Also, women more frequently have stable angina than MI as their first manifestation of CAD when compared with men[115].

Gender difference in MMPs

Plasma levels of MMP-3 were significantly lower in women as compared to men in normal subjects, and similar non-significant trends were seen in patients with RA, osteoarthritis, systemic lupus erythematosus and scleroderma[49]. On the other hand, no association of serum concentrations of MMP-3 or MMP-9 with gender was found in a study of hyperlipidemic subjects[61]. Circulating plasma concentrations of MMP-2 and MMP-9 did not show any gender-specific difference in a study of healthy subjects[129] whereas plasma MMP-9 concentration turned out to be higher in women than men amongst patients with stable CAD[124].

Little is known about possible sex hormone effects on MMPs. 17β-oestradiol decreased the expression of MMP-1 but had no effect on MMP-3 or MMP-13 expression in osteoarthritic chondrocytes[130]. A recent study of human aortic SMCs showed that testosterone increased MMP-3 protein two-fold compared with control cells and cells cultured with both oestrogen and progesterone[131]. The increase was mainly due to increased MMP-3 transcription. In contrast, MMP-2 gene and protein expression were unchanged despite the influence of sex steroids. Furthermore, the collagen deposition was reduced and elastin deposition increased in the presence of oestrogen and progesterone in combination, resulting in a highly increased elastin/collagen ratio[131]. Macrophage infiltration and MMP-9 expression were decreased by oestradiol in a rat model of AAA[132].

HYPOTHESIS AND AIMS

Hypothesis

Inflammatory and genetic mechanisms influence the synthesis and degradation of arterial vessel wall matrix through the effects of matrix metalloproteinases, and have a prominent role in initiation, progression and rupture of atherosclerotic plaques.

Aims

- to study the effect of the MMP-3 -1612 5A/6A promoter polymorphism on circulating levels of MMP-3 and angiographically assessed CAD
- to explore the serum MMP-3 concentration in relation to the acute stage of MI
- to identify functional polymorphisms of the MMP-1 gene that might influence the expression and plasma MMP-1 concentration, and the risk of MI
- to study circulating levels of inflammatory cytokines in postinfarction patients, and their relations to the levels of MMP-3 and MMP-1

SUBJECTS AND METHODS

Study populations

The present thesis is based on three different study populations, two of which were collected by our group in Stockholm (paper I, II, III and IV) and the third explored in collaboration with our co-workers in Southampton (paper III).

SCARF (papers I, III and IV)

The Stockholm Coronary Artery Risk Factor (SCARF) study was designed for studies of genetic, biochemical and environmental risk factors predisposing to MI. During the period of January 1996 to December 2000, all patients less than 60 years of age, who were admitted for acute MI to the coronary care units of the three hospitals in the northern part of Stockholm (Danderyd Hospital, Karolinska Hospital and Norrtälje Hospital) were screened for inclusion in the SCARF study (Figure 1). A total of 755 patients were considered, of whom 433 patients entered the study. Of the remaining 322 patients, 179 met one or more predefined exclusion criteria (previous MI (n=102), type 1 diabetes mellitus (n=23), renal insufficiency defined as serum creatinine >200 µmol/L (n=9), any chronic inflammatory disease (n=13), drug addiction (n=10), psychiatric disease (n=3) or unwillingness to enrol in the study (n=19)) and 143 patients were not included for logistic and psychosocial reasons, resulting in a participation rate of eligible patients of 76%.

Of the 433 patients entering the study (Figure 2), 46 patients did not complete the programme; 19 withdraw their consent, 19 were excluded due to occurrence of concomitant disease (n=12) or protocol violation (n=7), five patients failed to complete the programme and three died. Thus, 387 patients completed the programme and for each patient included, a sex- and age-matched healthy control person was recruited from the general population of the same county.

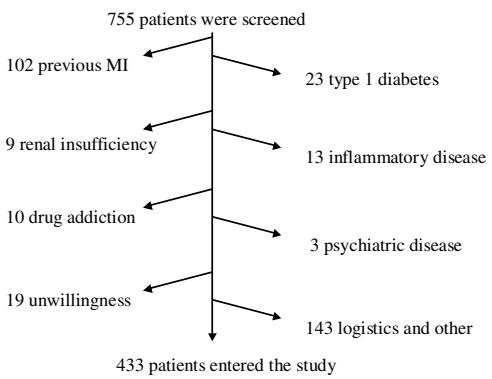

Figure 1: Patients screened for the SCARF study. Numbers and reasons for exclusion.

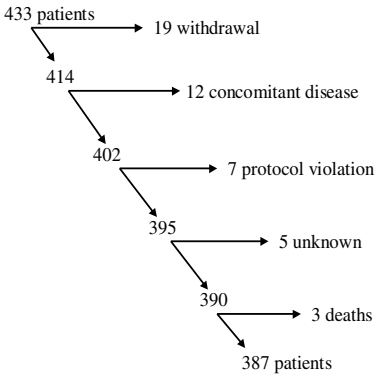

Figure 2: Patients not completing the SCARF study protocol. Numbers and reasons for drop-out.

Three months after the index cardiac event, patients and control subjects were interviewed about background facts, such as ethnicity, social situation, lifestyle characteristics, medical history and medication, and a medical examination was performed. Blood samples were drawn on the same occasion under fasting conditions. Basic characteristics and biochemical analyses are summarized in Table 5.

All patients included at the Danderyd and Norrtälje hospitals (n=269) were offered coronary angiography, of whom 243 accepted to be included in the coronary angiography substudy. Coronary angiography was performed, if needed for clinical reasons, during the initial hospital stay (n=35), or otherwise three months later (n=208).

The study was approved by the Ethics Committee of the Karolinska University Hospital, and conducted in agreement with the Declaration of Helsinki. All subjects gave their informed consent to participation.

TREOC (paper II)

During the time period of 1991 through 1995, 222 consecutive patients admitted to the coronary care unit at the Danderyd hospital with STEMI treated with thrombolysis were enrolled in the Thrombolysis and REOCclusion (TREOC) study, a longitudinal cohort study with a two year follow-up[133]. Three hundred and eighty patients were eligible for the study thus resulting in a participation rate of 58%. Reasons for patients not entering the study were mainly logistic, but also cardiogenic shock, unwillingness and alcohol abuse. However, for a minor part (22 patients) the reason could not be identified. All patients were below 75 years of age and 76% were men. Time elapsed since the onset of symptoms was less than 12 hours and diagnosis of STEMI was made by conventional electrocardiographic findings. The majority of the patients (89,3%) received streptokinase as thrombolytic therapy and the remaining patients were given front-loaded recombinant tissue-type plasminogen activator.

Table 5: Basic characteristics of patients and control subjects in SCARF

	Patients (n=387)	**Controls** (n=387)	**p-value**
Age	54 (49-57)	54 (49-57)	
Male (%)	82	82	
Smokers (%)	50	25	< 0.0001
Family history of CHD (%)	42	21	< 0.0001
Diabetes (%)	11	0	< 0.0001
Hypertension (%)	34	6	< 0.0001
Hyperlipidemia (%)	70	16	< 0.0001
BMI (kg/m^2)	26.8 (24.7-29.7)	25.6 (23.8-27.8)	< 0.0001
SBP (mmHg)	130 (118-140)	128 (118-140)	ns
DBP (mmHg)	80 (75-88)	80 (78-88)	ns
Glucose (mmol/L)	5.3 (5.0-5.9)	4.8 (4.6-5.2)	< 0.0001
Plasma cholesterol (mmol/L)	5.0 (4.3-5.7)	5.4 (4.7-6.1)	< 0.0001
Plasma triglycerides (mmol/L)	1.6 (1.2-2.2)	1.2 (0.8-1.6)	< 0.0001
LDL-cholesterol (mmol/L)	3.2 (2.5-3.9)	3.4 (2.9-4.2)	< 0.0001
HDL-cholesterol (mmol/L)	1.1 (0.9-1.3)	1.4 (1.1-1.6)	< 0.0001
Insulin (pmol/L)	47.0 (32.0-69.0)	36.0 (27.5-50.5)	< 0.0001
Proinsulin (pmol/L)	5.1 (3.4-7.5)	3.5 (2.6-5.4)	< 0.0001
CRP (mg/L)	1.5 (0.7-3.4)	1.0 (0.5-1.8)	< 0.0001
Cystatin C (mg/L)	0.81 (0.73-0.93)	0.83 (0.77-0.90)	ns
Fibrinogen (g/L)	3.8 (3.3-4.4)	3.5 (3.1-4.0)	< 0.0001
PAI-1 (IU/mL)	12.6 (4.8-22.8)	7.5 (3.0-17.6)	< 0.0001

Values are median (interquartile range) or percentage. CHD, coronary heart disease; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low density lipoprotein; HDL, high density lipoprotein; CRP, C-reactive protein; PAI-1, plasminogen activator inhibitor-1.

Blood samples were drawn at admission (within 12 hours of onset of symptoms) and 48 hours, 6 days and 3, 6, 12 and 24 months after the admission. Coronary angiography was performed before discharge and at 6 months after the acute event. Patients were excluded from further blood sampling if they had a second MI or if they were subjected to PCI or CABG during follow-up. A total of 14 patients died in the course of follow-up whereas 109 patients completed the two-year program. One hundred and sixty-four patients were event-free survivors and had blood samples stored for DNA sequencing and for serum analyses from all investigations up to the three months control and formed the basis for the study included in this thesis. Two hundred and five patients had DNA isolated at inclusion, whereas 41 were either excluded due to events (n=35) or lost to follow-up (n=6). Basic characteristics of patients, both those included and excluded in this study, are presented in Table 6.

All patients gave informed consent to participation and the Ethics Committee of the Karolinska University Hospital approved the study.

SAS, the British cohort (paper III)

The Southampton Atherosclerosis Study (SAS) conducted at the Wessex Cardiothoracic Unit, Southampton General Hospital, included 1501 consecutive patients undergoing diagnostic or interventional coronary angiography[134]. All patients with significant CAD, defined as at least one coronary artery diameter stenosis of >50%, were included in this study. The participants were divided into two groups by history of MI; 639 patients had suffered an MI, and 538 had not, constituting the MI and non-MI groups respectively. Clinical and demographic data were recorded and serum lipids measured. MI was diagnosed according to standard clinical criteria including electrocardiographic and/or enzymatic changes. Characteristics of MI and non-MI groups are given in Table 7.

The study was approved by the South and West Local Research Ethics Committee, and all subjects gave written consent.

Table 6: Basic characteristics of patients at baseline in TREOC

	Included (n=164)	**Excluded** (n=41)
Age (years)	60 (10)	64 (9)*
Males	125 (76)	35 (85)
Current smokers	75 (46)	10 (24)*
Family history of CHD	103 (63)	20 (49)
Previous MI	20 (12)	12 (30)*
Diabetes	18 (11)	3 (7)
Hypertension	52 (32)	10 (24)
SBP (mmHg)	152 (26)	149 (30)
DBP (mmHg)	92 (15)	88 (14)
BMI (kg/m^2)	25.9 (3.7)	24.5 (3.1)*
Plasma cholesterol (mmol/L)	5.8 (0.9)	5.8 (0.9)
LDL cholesterol (mmol/L)	4.0 (0.9)	4.1 (0.9)
HDL cholesterol (mmol/L)	1.1 (0.3)	1.0 (0.2)
Plasma triglycerides (mmol/L)†	1.5 (1.1-2.2)	1.7 (1.2-2.5)

Values are mean (+/-SD) or number of patients in group (%), †for skewed data median (interquartile range), * $p<0.05$ by student t-test or chi-square, CHD, coronary heart disease; MI, myocardial infarction; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; HDL, high density lipoprotein

Table 7. Basic characteristics of SAS

	MI group (n=639)	Non-MI group (n=538)	p-value
Age (years)	62.9 (10.0)	63.8 (9.9)	ns
Gender (male, %)	79	73	<0.05
Body mass index (kg/m^2)	27.6 (4.3)	27.5 (4.1)	ns
Smokers (current and previous, %)	77	71	<0.05
Statin treatment (%)	60	52	<0.01
Plasma cholesterol (mmol/L)	5.0 (1.0)	5.3 (1.1)	<0.01
HDL cholesterol (mmol/L)	1.2 (0.3)	1.3 (0.3)	<0.05
Plasma triglyceride (mmol/L)	1.8 (1.2)	1.9 (1.1)	ns
Hypertension (%)	42	49	<0.05
Type 2 diabetes (%)	10	10	ns

Mean (standard deviation) are shown for quantitative variables. HDL, high-density lipoprotein

Biochemical analyses

Fasting plasma concentrations of cholesterol and triglycerides and concentrations of cholesterol and triglycerides in very low density lipoprotein (VLDL), LDL and high density lipoprotein (HDL) were determined by a combination of preparative ultracentrifugation, precipitation of apo-B containing lipoproteins and lipid analyses[135].
Commercially available kits were used for analysis of insulin and proinsulin (ELISAs from DAKO Ltd, Cambridgeshire, UK), fibrinogen (clotting assay IL-Test Fibrinogen-C from Instrumentation Laboratory Co., Milan, Italy), and plasminogen activator inhibitor-1 (PAI-1) activity (Cromolize PAI-1 from Biopool International, Umeå, Sweden). High sensitivity CRP (hsCRP; N high sensitivity CRP) and cystatin C (N latex Cystatin C) were measured by particle-enhanced immunonephelometry with specific assays in the BN system (Dade Behring, Liederbach, Germany).

MMP quantification

MMP levels were measured using sandwich enzyme-linked immunosorbent assays (ELISA). ELISA is a robust and commonly used technique for quantification of proteins in body liquids and in cell culture supernatants. Briefly, there is a solid phase antibody to the MMP being measured, the study sample is added and the MMP attaches to the antibodies. After washing away the unbound material, a second MMP-antibody labelled with an enzyme (peroxidase) is added which binds to the MMP caught in the solid phase. After a second washing, a peroxidase substrate (tetramethylbenzidine) is added that generates a coloured product, the amount of which is proportional to the amount of MMP present in the study sample. The intensity of the colour is quantified by a spectrophotometric reading at 450 nm. A possible drawback, depending on the antibody, in the MMP setting is that it does not discriminate the different forms of MMP present in the circulation (pro-MMP, active MMP and MMP bound to TIMPs) resulting in quantification of total MMP.

MMP-3 was quantified in serum samples with an immunoassay from R&D Systems (Abingdon, UK). The MMP-3 inter-assay coefficient of variation (CV) for a control sample was 8.9%. The lower detection level is 0.009 ng/mL according to the manufacturer.

MMP-1 was quantified in plasma samples with an ELISA from GE Healthcare (Uppsala, Sweden) The MMP-1 inter-assay CV for a normal citrate plasma was 8%, and the intra-assay CV was always lower than 2%. Sensitivity level of measurement is 1.7 ng/mL according to the manufacturer.

Cytokines

Plasma concentrations of IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IFN-γ, TNF-α and MCP-1 were measured using a biochip array system based on standard sandwich and competitive immunoassay techniques (evidence®, Randox Laboratories Ltd., Co Antrim, UK)[136].

Genotyping

Genotyping can be performed using a variety of methods, all of which are based on four general mechanisms, which are: allele-specific hybridisation, nucleotide incorporation, oligonucleotide ligation and invasive cleavage[137]. In this thesis the allele-specific nucleotide incorporation principle has been utilized in the pyrosequencing method. In addition, restriction fragment length polymorphism (RFLP) analysis, the classical genotyping method, was used in paper III.

Pyrosequencing (papers I and II)

Pyrosequencing is a sequencing technique developed in Sweden and first described by Nyrén[138-140]. This technique is a suitable method to detect SNPs and insertion/deletion polymorphisms as the sequencing of the DNA is done at the polymorphic site. The nucleotides are added one at a time in a pre-specified order, exclusive for the SNP studied, and they are incorporated into the extending DNA-primer if suitable. On incorporation a pyrophosphate is released which by a luciferase produces a light signal that is detected and presented as a peak in the pyrogram (Figure 3). The height of the peak is proportional to the amount of incorporated nucleotide. This method was used in genotyping for the MMP-3 -1612 5A/6A promoter polymorphism in papers I and II.

DNA was prepared from peripheral blood cells using a genomic DNA isolation kit (Qiagen Inc, Valentia, CA) and stored frozen in 96-well arrays. For determination of -1612 5A/6A MMP-3 genotype, DNA was amplified by a nested polymerase chain reaction (PCR) to get a clean PCR product for the pyrosequencing analyses. The primers used in the outer PCR were forward primer 5'-CTCCACTGTTTCTTCCTGGAATTC-3' and reverse primer 5'-CAAGTGATTCTCCTGCCT CAACCT-3'. The inner PCR used the forward primer 5'-CTCTGTTCTCCTTGTCCTCATATC-3' and the biotinylated reverse primer 5'-GGCAC CTGGCCTAAAGACATTTTA-3'.

The nucleotide sequence was then identified by pyrosequencing™ using the PSQ™ 96 System with PSQ™ 96 SNP Reagent Kit in paper I, and the PSQ™ 96 MA System with PSQ™ 96 MA Pyro Gold Reagents in paper II (Pyrosequencing AB, Uppsala, Sweden). The primer used for pyrosequencing was 5'-AATCAGGACAAGACATG GTTTTT-3'. The dispensation order of the nucleotides was ACTACATCA. The resulting pyrograms of the MMP-3 -1612 5A/6A promoter polymorphism are presented in Figure 3.

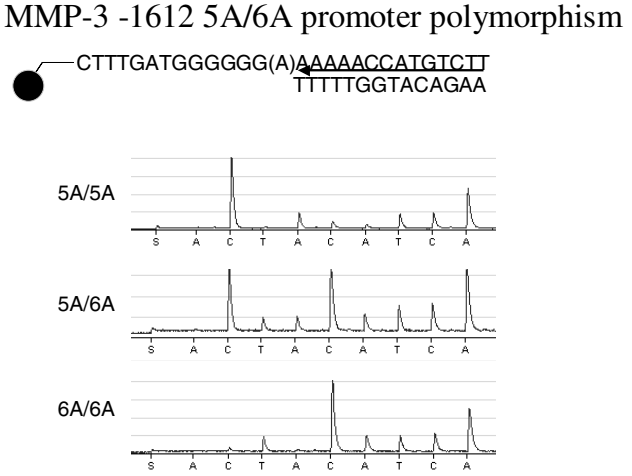

Figure 3: The Pyrogram™, an illustration of pyrosequencing results for each genotype of the MMP-3 –1612 5A/6A promoter polymorphism. The added nucleotide sequence is on the X-axis. The signal peak reflects the number of incorporated nucleotides in the complementary DNA strand for the different genotypes. The dispensation order of the nucleotides has been designed to create an allele-specific out-of-phase shift of the sequencing. When adding the first C, it is incorporated only in the 5A-allele, giving the intense signal from the 5A-allele, as the 6A-allele requires an additional T before any C can be incorporated.

RFLP analysis (paper III)

Analysing RFLP is the prototypic genotyping method for SNPs. After amplification of the DNA region of interest by PCR, a restriction enzyme (or restriction nuclease) is added which specifically recognizes one of the two allele variants of the SNP and splices the DNA on recognition. The product is then separated by gel electrophoresis and visualized by fluorescent staining. Heterozygous samples appear as two bands whereas homozygous samples present either of the two bands.

For each of the seven identified MMP-1 promoter polymorphisms, a DNA sequence containing the polymorphic site was amplified by PCR using predefined primers. The amplicons were digested with an appropriate restriction endonuclease for each SNP; those were Xmn I (for -1607 G/GG), Hind III (for -839 G/A), Msp I (for -755 G/T), Kpn I (for -519 A/G), Ban I (for -422 T/A), Afl II (for -340 T/C), and Hae III (for -320 T/C), respectively. Digests were subjected to gel electrophoresis, and DNA was detected by post staining of the gel with Vistra Green in the SAS patients and ethidium-bromide in the SCARF cohort, and visualized using a fluoroimager to determine genotypes.

Genotyping for all seven polymorphisms in the SAS patients was performed at the Human Genetics Division, School of Medicine, University of Southampton, UK, whereas in the SCARF cohort only the -519 A/G and -340 T/C genotypes were analysed, and this was done at King Gustaf V Research Institute, Karolinska Institutet at the Karolinska University Hospital.

Coronary angiography

Coronary angiography has for a long time been the golden standard for diagnosing CAD and measuring extent and severity of the disease. The cardiac catheterisation method rendered Werner Forssmann, André Cournand and Dickinson Richard the Nobel Prize in 1956 and the selective coronary angiography came into routine use in the 60's and 70's[141]. Coronary angiography is a radioangiographic method using iodine based radioopaque contrast medium to visualize the lumen of the arteries. A stenosis appears as narrowing of the lumen of the coronary artery tree or sudden interruption of the artery in case of occlusions, a common finding in STEMI. In earlier years, the images were stored on 35 mm cine films but nowadays they are collected digitally and stored on a server or on compact discs. The digital images can then be interpreted by computerized algorithms.

Despite the usefulness of coronary angiography in detecting stenoses and need for revascularization, a crucial limitation of this method is the "lumenography", the two-dimensional luminal imaging. From angiography you cannot tell anything about the vessel wall or the composition of the plaques intruding into the lumen. In fact, it is shown that substantial alterations in the vessel wall can occur without any change to the inner lumen surface. Thus, the vessel may look perfectly normal on the angiographic images. Intravascular ultrasound (IVUS) is a method imaging the vessel wall cross-sectionally, in a histology-like fashion, and has been developed in the late 80's. The method is mainly used for research. IVUS makes it possible, at least to some extent, to discriminate between lipid-rich and fibrous plaques *in vivo*.

QCA

Quantitative coronary angiography (QCA) is a computerized method designed for analysing coronary angiographies (Figure 4). In this thesis (papers I and IV), the Medis QCA-CMS system developed in Leiden, the Netherlands, has been used. The intra- and inter-observer variability of QCA has been addressed in several papers. The QCA method has proved to be superior to visual methods and hand-held calipers[142]. Validation of the QCA-CMS system has been performed by several authors and the intra-measurement variability of the diameter stenosis was found to be 0.5-1.0 ± 2.5-3.7% (± standard deviation (SD)) and the SD of minimal lumen diameter (MLD) measurements was 0.07-0.094 mm[143-145].

In SCARF

Coronary angiograms were obtained, after intracoronary administration of glycerylnitrate (0.1 mg/mL, 2.5 mL), using the Philips Integris H3000 angiographic system. The first 200 examinations were stored on 36 mm cine film and the remaining

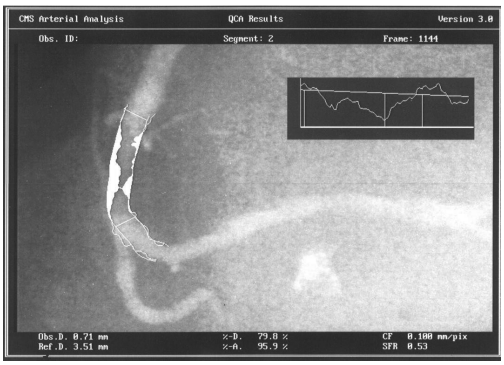

Figure 4: QCA image of a stenosis in the right coronary artery. The true borders of the lumen and the computer estimated reference vessel borders are outlined. The filled area is the plaque area. %-D, diameter stenosis; %-A, area stenosis.

43 on CD-ROM. Angiograms were analysed by QCA (Medis QCA-CMS system). The coronary artery tree was divided into 15 segments according to American Heart Association (AHA) guidelines[146]. In each segment, MLD, reference diameter, % diameter stenosis, mean segment diameter (MSD), segment length, plaque area, segment area and number of significant (>50%) stenoses were registered.

In TREOC

Coronary angiography was performed after administration of 0.5 mg sublingual nitroglycerine by using the transfemoral technique and 7F Judkin diagnostic catheters. The angiographies were stored on 35 mm cinefilm. Severity of CAD was quantified as number of major coronary arteries with at least one stenosis of more than 50% diameter reduction (0-3 vessel disease) as assessed by one experienced observer who was blinded to the clinical characteristics and outcome of the patient.

Functional characterization of promoter activity

EMSA

Electrophoretic mobility-shift assays (EMSAs) were performed in paper III to investigate whether the different alleles (-519 A/G and -340 T/C) of the MMP-1 haplotype studied were recognized by nuclear proteins, and if so, whether the alleles differed in the affinity to nuclear proteins. For each allele, a double-stranded oligonucleotide probe of 24 bases up- and downstream of the respective polymorphism was created and labelled with radioactive ^{32}P at the 5' terminus. The sequences of the probes were 5'-GCCATGGTGCTATCGC AATAGGGT**A/G**CCAGGCAGCTTAACAAAG GCAGAA-3'and 5'-GGTGTGTGGAGAAACC TGTAGCAC**C/T**TTATGACCATCAGAACCA GTCTTT-3' (polymorphisms in bold). The labelled probe was incubated with nuclear protein extracts from cultured human macrophages derived from THP-1 cells, either alone, or with unlabeled specific or non-specific competitor. DNA with bound nuclear protein was separated from unbound DNA on a polyacrylamide gel by electrophoresis. The gel was read by autoradiography using a phosphoimager (Fuji). Three independent experiments were performed for each polymorphism.

Transient transfection and reporter assays

Transient transfection and reporter assays were performed in paper III to evaluate whether the different haplotypes influenced the MMP-1 promoter activity. Genomic DNA from the nine most common haplotypes derived from the initial sequencing analysis in paper III were used as templates for generating the MMP-1 promoters by PCR, and subsequently cloned into a TOPO PCR cloning vector (Invitrogen). The promoters were cloned from -1870bp to +42bp relative to the transcriptional start site. The cloned MMP-1 promoter was sequenced to verify that there was no misincorporation during PCR. The created promoters were then subcloned into a plasmid (pGL3-Basic Vector, Promega) containing a firefly luciferase reporter gene. The resultant construct was mixed with a plasmid (pRL-TK, Promega) containing a *renilla* luciferase gene under the

control of a thymidine kinase promoter. Lipofection with FuGENE 6 transfection reagent was used to transfect cultured THP-1 human monocytic cells with the respective plasmid mix. The transfected cells were treated with phorbol 12-myristate 13-acetate (PMA) to induce differentiation into macrophages. At 36 hours after transfection, the cells were lysed and the activities of the firefly luciferase and *renilla* luciferase in the lysates were measured with the use of a dual-luciferase assay kit (Promega). The ratio of firefly luciferase level to *renilla* luciferase level was used as a measure of the MMP-1 gene promoter activity. Three independent experiments in duplicate were performed for each construct.

Real-Time RT-PCR

In the real-time reverse transcriptase (RT)-PCR experiment performed in paper III, RNA was extracted from atherosclerotic plaques removed from patients undergoing carotid endarterectomy. RNA was reverse transcribed to complementary DNA (cDNA). Real-time PCR was performed in duplicates in an ABI Prism 7700 Sequence Detection System. PCR primers were designed with the use of the Primer Express program (Applied Biosystems), with the forward and reserve primers placed in different exons. The MMP-1 real-time RT-PCR results of different samples were standardized for the amounts of RNA template and efficiencies of reverse transcription, using the housekeeping gene 36B4 (acidic ribosomal phosphoprotein PO subtype) as a reference, and relative quantification was done by use of the $2^{-\Delta\Delta CT}$ method[147]. The haplotype effects of the MMP-1 -519 A/G and -314 C/T polymorphisms on MMP-1 mRNA levels were analysed using the THESIAS program.

Statistical analyses

Statistical analyses were performed by using the STATISTICA software, version 6.0 in paper I and version 7.1 in papers II and IV (StatSoft inc, Tulsa, OK). Results are presented either as mean ± SD or as median, with the interquartile range in brackets. For quantitative variables with a skewed distribution, data were log-transformed to achieve a normal distribution before comparisons were done. Comparisons between two groups were made by the χ^2 test for categorical variables and the unpaired Student's t-test for continuous variables.

A one-way analysis of variance (ANOVA) was performed with the Scheffé post-hoc test and planned comparisons when analysing serum concentrations of MMP-3 in relation to genotype and a factorial ANOVA was used when comparing gender and patient and control groups. In paper II, relationships of genotype or CAD severity groups to serum MMP-3 concentration were assessed by ANOVA. For comparisons between different time points repeated measures ANOVA was used.

In paper IV, cytokine and MMP-1 data were extremely skewed, and the non-parametric Mann-Whitney U test was used for case-control comparisons whereas Spearman rank correlation coefficients were computed to estimate interrelations. A second analysis was done based on dichotomised cytokine data: values above as opposed to below detection level. The χ^2 test was then used for patient-control comparisons.

Associations of individual polymorphisms with MI in papers I and III were examined by χ^2 analysis. In the haplotype analyses in paper III, a systematic analysis of all possible combinations of polymorphisms to select the most informative haplotype configuration in terms of predicting disease status was performed using a method that calculates the Akaike's Information Criterion (AIC) values for each haplotype model and then subtracts the minimum AIC value obtained for each model over all models explored, giving a rescaled AIC value for each haplotype model[148]. The model with a rescaled AIC ≤ 2 and including the fewest polymorphisms is considered the best model. Because the choice of model is based on AIC values, it circumvents problems associated with methods based on null-hypothesis testing, such as the requirement of multiple testing correction. After identifying the best model, the haplotype effects of the polymorphisms in this model on MI risk were analysed using the THESIAS program (www.genecanvas.org), which implements the stochastic-EM (Expectation-Maximization) algorithm[149]. Haplotype effects on MI risk were adjusted for age, gender, body mass index, smoking, cholesterol levels, statin treatment, hypertension and diabetes.

RESULTS

MMP-3 and MMP-1 concentrations in postinfarction patients (papers I and IV)

Serum concentrations of MMP-3, in the SCARF study (paper I), were lower amongst patients than controls (16.2 ±6.9 vs. 19.2 ±8.2 ng/mL, p<0.0001, Figure 5). Exclusion of subjects on statin treatment (35% of patients) did not change this pattern (16.4 ±7.3 vs. 19.2 ±8.2 ng/mL, p<0.0001). In contrast, there was no difference in plasma MMP-1 concentration between patients and controls (4.29 [3.98-4.51] vs. 4.25 [3.98-4.54] ng/mL, p=0.70). Statin treatment did not appear to influence the MMP-1 concentration. In addition, there were no differences in either MMP-3 or MMP-1 concentrations between patients on different treatment, e.g. with and without betablockers and angiotensin converting enzyme (ACE) inhibitors, respectively. There was no association between any medical treatment at inclusion and serum MMP-3 concentrations.

Relationships of MMP-3 and MMP-1 concentrations to established risk indicators for CAD were explored separately in patients and controls. In control subjects, but not in patients, serum MMP-3 concentrations correlated significantly with both systolic (r=0.14, p<0.01) and diastolic (r=0.18, p<0.001) blood pressure, blood glucose (r=0.11, p<0.05) and plasma triglycerides (r=0.13, p<0.05). In patients, on the other hand, a negative correlation was seen between serum MMP-3 concentration and BMI (r=-0.16, p<0.01). Furthermore, serum MMP-3 concentrations correlated significantly with cystatin C in both groups but the correlation was stronger in patients (r=0.27 p<0.0001) than in controls (r=0.11 p<0.05).

The plasma MMP-1 concentration correlated with BMI (r_s=0.14, p<0.05), insulin (r_s=0.30, p<0.05) cystatin C (r_s=0.18, p<0.05) and PAI-1 (r_s=0.30, p<0.05) in the patient group, whereas in the control group the plasma MMP-1 concentration correlated only with insulin (r_s=0.25, p<0.05) and PAI-1 (r_s=0.17, p<0.05).

MMP-3 concentration in acute MI (paper II)

In the SCARF study, blood samples were collected three months after the acute event. Concerns could be raised that the MMP-3 level might be altered in the very acute stage of MI, possibly higher, and then, in the recovery phase, return to a lower level. To elucidate this question we analysed the MMP-3 concentrations also in a group of patients suffering STEMI (the TREOC study), in which we had access to blood samples drawn at several time points during and after the acute stage of MI. Interestingly, in the TREOC study we found a significant increase in serum MMP-3 concentration at three months compared with at admission and at 48 hours (19.5 [14.4-24.7] vs. 15.5 [10.5-21.8] ng/mL, p<0.001 and 14.7 [9.9-23.8] ng/mL, p<0.001, respectively). Furthermore, there was no significant change in serum MMP-3 concentration during the first 48 hours.

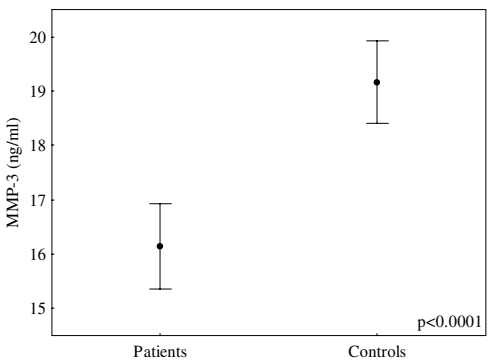

Figure 5: Serum MMP-3 concentration in patients and controls in the SCARF study (paper I). Mean and 95% confidence intervals denoted by bars.

MMP-3 and MMP-1 concentrations and angiographic score (papers I, II and IV)

In the SCARF study there were no significant correlations between serum MMP-3 concentration and angiography measurements obtained by QCA in the entire patient group, or amongst male patients separately. In female patients, however, a negative correlation (r= -0.39, p<0.05) was found with plaque area. The corresponding association in male patients was non-significant (r=0.02).

The TREOC study, on the other hand, showed that the serum MMP-3 concentration at three months increased with severity of CAD, and the differences were statistically significant between patients with 1- and 2-vessel disease (p=0.02), and patients with 1- and 3-vessel disease (p<0.001, Figure 6).

There were no differences in extension and severity of CAD according to MMP-3 -1612 5A/6A genotype, neither in SCARF, nor in the TREOC study.

Plasma MMP-1 concentrations showed no significant correlations with the QCA measurements of CAD severity amongst patients.

MMP-3 genotype and MMP-3 concentration (papers I and II)

In the SCARF study population as a whole, patients and controls analysed together, the distribution of MMP-3 -1612 5A/6A genotype was 26% (5A/5A), 46% (5A/6A) and 28% (6A/6A), respectively, giving a 6A-allele frequency of 0.51. There was a clear association between MMP-3 -1612 5A/6A genotype and serum MMP-3 concentration, with the presence of one or two copies of the 6A-allele being associated with a graded increase in serum MMP-3 concentration. Accordingly, in all participants analysed together, serum MMP-3 concentrations were lowest in 5A/5A homozygotes (13.3 ±5.7 ng/mL), intermediate in 5A/6A heterozygous individuals (17.5 ±6.5 ng/mL) and highest amongst 6A/6A homozygotes (22.1 ±8.7 ng/mL, p<0.0001). The allelic dose-dependent effect was seen in both groups and was even more pronounced among control subjects (Figure 7).

The clear allelic dose-dependent result was surprising as we had expected the 6A/6A homozygotes to have the lowest levels according to *in vitro* experiments showing the 6A-allele to be associated with reduced expression[48]. To confirm this result, we performed this analysis in the second cohort of MI patients, the TREOC study.

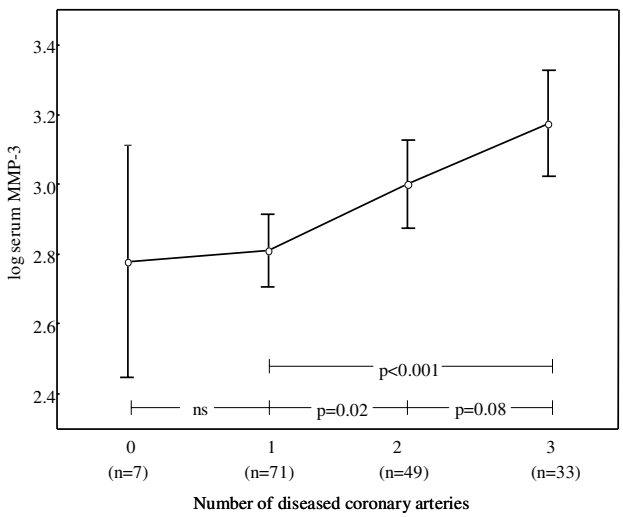

Figure 6: Serum MMP-3 concentration increases with the number of diseased vessels in the TREOC study (paper II). Logarithm of serum MMP-3 at the three months follow-up visit on Y-axis. Mean and 95% confidence interval denoted by bars.

In the TREOC study, a total of 43 patients (26%) were 5A/5A homozygotes, 79 (48%) were heterozygotes and 42 (26%) were 6A/6A homozygotes, giving a 6A-allele frequency of 0.50. The graded increase in serum MMP-3 concentration according to number of 6A-alleles was confirmed at all time points ($p<0.001$ in ANOVA, Figure 8). Serum MMP-3 distribution according to genotype at three months revealed 5A homozygotes to have the lowest levels (14.1 [10.2-18.8] ng/mL), heterozygotes to have intermediate levels (19.6 [15.0-24.4] ng/mL) and 6A homozygotes to have the highest serum MMP-3 levels (24.0 [20.1-32.3] ng/mL, $p<0.001$ for comparisons between all groups).

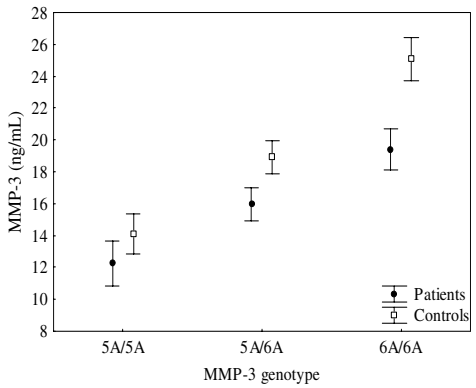

Figure 7: Serum MMP-3 concentration increases with the number of 6A-alleles both in patients and controls in the SCARF study (paper I). Mean and 95% confidence intervals denoted by bars.

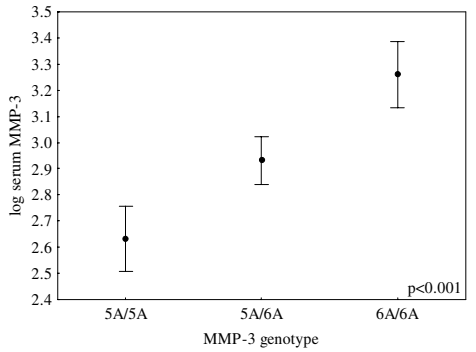

Figure 8: Serum MMP-3 concentration increases with number of 6A-alleles in the TREOC study (paper II). Logarithm of serum MMP-3 at the three months follow-up visit on Y-axis. Mean and 95% confidence intervals denoted by bars.

Gender and MMP-3 and MMP-1 concentrations (papers I, II and IV)

There was a striking gender difference predominantly in MMP-3 but also in MMP-1 concentrations in both cohorts analysed. In the SCARF study, a gender difference in serum MMP-3 concentrations was present (Figure 9) both among patients and control subjects (17.5 ±6.7 ng/mL in male patients vs. 9.1 ±3.3 ng/mL in female patients, $p<0.0001$; 21.0 ±7.6 ng/mL in male controls vs. 10.1 ±3.5 ng/mL in female controls, $p<0.0001$). Gender explained 22% of the difference in serum MMP-3 concentration using a stepwise regression model including the genotype effect. The gender difference in serum MMP-3 concentration was confirmed in the TREOC study where female patients had significantly lower serum concentrations of MMP-3 than male patients at the three months follow-up visit (14.8 [9.4-20.8] vs. 19.9 [16.0-26.9] ng/mL, $p<0.0001$, Figure 10). The effect of gender was significant at all three time points in TREOC and for all MMP-3 -1612 5A/6A genotypes in both SCARF and TREOC.

Plasma levels of MMP-1 exhibited a gender difference as well. Women in the SCARF study had lower MMP-1 concentration than men when pooling patients and controls (4.16 [3.90-4.46] vs. 4.29 [4.00-4.54] ng/mL, $p<0.05$). Analysing the two groups separately revealed the gender difference to be statistically significant in the control group (4.08 [3.91-4.38] ng/mL in women vs. 4.26 [4.00-4.56] ng/mL in men, $p<0.05$) but not in the patient group (4.19 [3.89-4.53] ng/mL in women vs. 4.31 [4.00-4.50] ng/mL in men, $p=0.20$).

Identification of polymorphisms in the MMP-1 gene (paper III)

To search for naturally occurring, common sequence variants in the MMP-1 gene, we sequenced the promoter, exons, and intron-exon junctions of the gene in 30 unrelated Caucasian individuals. In the promoter region, SNPs were identified at positions -1607 (G/GG, i.e. G insertion/deletion), -839 (G/A), -755 (G/T), -519 (A/G), -422 (T/A), -340 (T/C) and -320 (T/C). In the coding region, only two polymorphisms were identified, neither

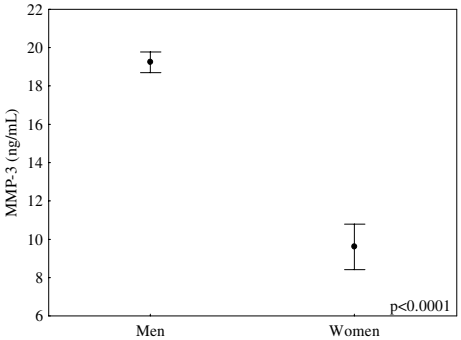

Figure 9: Higher serum MMP-3 concentration in men than in women in the SCARF study. Mean and 95% confidence intervals denoted by bars.

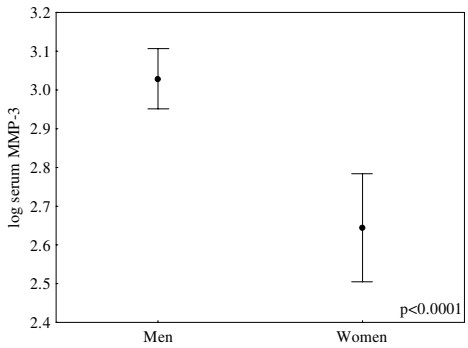

Figure 10: Higher serum MMP-3 concentration in men than in women in the TREOC study. Logarithm of serum MMP-3 at the three months follow-up visit on Y-axis. Mean and 95% confidence intervals denoted by bars.

of which generated changes in the amino acid sequence. The subsequent studies were therefore focused on the promoter polymorphisms.

MMP-1 haplotype effect on risk of MI (paper III)

No significant difference in genotype distribution of individual polymorphisms was detected between the two groups in the SAS cohort. A systematic analysis of all possible combinations of the polymorphisms in relation to MI was performed, and the analysis showed that the best model in terms of predicting MI status was one that consisted of the -519 A/G and -340 T/C polymorphisms. Haplotype analysis based on these two polymorphisms showed that the haplotype frequencies were significantly different between the MI and non-MI groups (χ^2=16.48 with 3 df, p<0.001, Table 8). Compared with the A_{-519}-T_{-340} haplotype, both the A_{-519}-C_{-340} and G_{-519}-T_{-340} haplotypes conferred a protective effect against MI (OR=0.68 [0.52–0.88], p=0.004, for A_{-519}-C_{-340}; OR=0.71 [0.56–0.89], p=0.003 for G_{-519}-T_{-340}, the common OR associated with these two haplotypes being OR=0.70 [0.57–0.86], p=0.0007, Table 8), whereas the G_{-519}-C_{-340} haplotype was associated with an increased risk of MI (OR=1.94 [1.15–3.28], p=0.013, Table 8).

To verify these findings, the SCARF cohort was genotyped for the two polymorphisms included in the haplotype analysis. The result confirmed a protective effect of the A_{-519}-C_{-340} and G_{-519}-T_{-340} haplotypes (OR=0.72 [0.54–0.94], p=0.02, for A_{-519}-C_{-340} versus A_{-519}-T_{-340}; OR=0.68 [0.52–0.89], p=0.005, for G_{-519}-T_{-340} versus A_{-519}-T_{-340}; and OR=0.70 [0.55-0.89], p=0.003, for A_{-519}-C_{-340} + G_{-519}-T_{-340} versus A_{-519}-T_{-340}; Table 8), and an increased risk for the G_{-519}–C_{-340} haplotype (OR=1.54 [0.97–2.46], p=0.07, for G_{-519}–C_{-340} versus A_{-519}-T_{-340}, Table 8).

Experimental evaluation of MMP-1 promoter haplotypes (paper III)

Effects of MMP-1 polymorphisms on nuclear protein binding

The EMSAs for the -519 A/G and -340 T/C polymorphisms indicated the presence of specific DNA-nuclear protein interactions for both polymorphisms. For the -519 A/G polymorphism, a DNA-protein complex was detected in assays using a probe corresponding to the A_{-519} allele but not in assays using a probe corresponding to the G_{-519} allele. For the -340 T/C polymorphism, a DNA-protein complex was detected when using a probe corresponding to the C_{-340} allele but not when using a probe corresponding to the T_{-340} allele. (Figure 2 in paper III)

Effects of MMP-1 haplotypes on promoter activity

To investigate whether the -519 A/G and -340 T/C polymorphisms had a functional effect, we examin-

Table 8. Haplotype effects of the MMP-1 -519 A/G and -340 T/C polymorphisms on risk of myocardial infarction

Haplotype	Haplotype frequency		Haplotype effects on risk of MI		Haplotype effects on risk of MI (homogeneous effects of A_{-519}-C_{-340} and G_{-519}-T_{-340})
SAS	**MI group**	**Control group**			
A_{-519}–T_{-340}	0.43	0.36	reference haplotype		reference haplotype
A_{-519}–C_{-340}	0.19	0.24	OR=0.68 (95% CI=0.52-0.88), p=0.004	}	OR=0.70 (95% CI=0.57–0.86), p=0.0007
G_{-519}–T_{-340}	0.31	0.37	OR=0.71 (95% CI=0.56-0.89), p=0.003		
G_{-519}–C_{-340}	0.07	0.03	OR=1.94 (95% CI=1.15-3.28), p=0.013		OR =1.93 (95% CI=1.14–3.26), p=0.014
SCARF					
A_{-519}–T_{-340}	0.40	0.34	reference haplotype		reference haplotype
A_{-519}–C_{-340}	0.22	0.27	OR=0.72 (95% CI=0.54–0.94), p=0.016	}	OR=0.70 (95% CI=0.55 - 0.89) p=0.003
G_{-519}–T_{-340}	0.27	0.34	OR=0.68 (95% CI=0.52–0.89), p=0.005		
G_{-519}–C_{-340}	0.11	0.05	OR=1.54 (95% CI=0.97–2.46), p=0.070		OR=1.53 (95% CI=0.96 - 2.44) p=0.074

OR, odds ratio; CI, confidence interval.
The analyses showed that there was no significant difference between the G_{-519}–T_{-340} and A_{-519}–C_{-340} haplotypes in relation to MI risk

ed the promoter activity of MMP-1 haplotypes in driving the expression of a luciferase reporter gene in transiently transfected THP-1 cell-derived human macrophages. Compared with the haplotypes encompassing both the A_{-519} and T_{-340} alleles, the haplotypes encompassing both the A_{-519} and C_{-340} alleles and those encompassing both the G_{-519} and T_{-340} alleles had lower promoter activity ($p<0.01$, Table 3 in paper III).

Haplotype effects on MMP-1 expression

MMP-1 mRNA levels in atherosclerotic plaques from patients with different haplotypes were estimated using the real-time RT-PCR method. The A_{-519}-C_{-340} and G_{-519}-T_{-340} haplotypes were associated with lower MMP-1 expression as compared with the A_{-519}-T_{-340} haplotype. The relative mRNA levels were 0.205 (95% CI 0.064-1.532) for A_{-519}-C_{-340} and 0.230 (95% CI 0.029-1.844) for G_{-519}-T_{-340} ($p=0.033$, Figure 3 in paper III). The rare G_{-519}-C_{-340} haplotype was not present in the patients studied.

Influence of circulating inflammatory cytokines (paper IV)

Blood samples for the biochip immunoassay were missing for 26 patients and 12 control subjects. Plasma concentrations of several cytokines (IL-2, IL-6, IL-8, and TNF-α) were higher in patients compared with controls (Table 9). More than 50% of the individuals had undetectable plasma levels of five of the cytokines (IL-1α, IL-1β, IL-4, IL-10 and IFN-γ). For evaluation of these cytokines we dichotomised the data as described in Subjects and methods, Statistical analyses. When comparing patients and controls, a trend towards a lower proportion of individuals with a detectable concentration of IL-10 was found in the patient group (39% vs. 46%, $p=0.086$) (Table 2 in paper IV). Conversely, a trend towards a higher proportion of individuals with detectable IL-1β levels was encountered in the patient group (50% vs. 44%, $p=0.082$).

MMP-3 showed weak inverse correlations with IL-6 ($r_S=-0.10$, $p<0.05$) and IL-8 ($r_S=-0.07$, $p<0.05$) when patients and controls were pooled, but the correlations were no longer statistically significant when the two groups were analysed separately (Table 10).

In contrast, the MMP-1 concentration was found to correlate more strongly and mostly positively with several cytokines (Table 10). The most consistent correlations were seen with IL-10 ($r_S=0.27$, $p<0.05$ in the combined sample) and IL-4 ($r_S=0.20$, $p<0.05$ in the combined sample). For IL-6 and TNF-α significant correlations were accounted for by the controls ($r_S=0.23$, $p<0.05$ for both). Weaker but still significant positive correlations were observed with IL-1α ($r_S=0.15$, $p<0.05$) and MCP-1 ($r_S=0.14$, $p<0.05$) in patients and with IL-1β ($r_S=0.14$, $p<0.05$) in controls. Also, a weak inverse correlation was found with IL-2 ($r_S=-0.16$, $p<0.05$) in the patient group. Of note, MMP-1 correlated inversely with IL-8 in patients ($r_S=-0.15$, $p<0.05$) but positively in controls ($r_S=0.11$, $p<0.05$).

Table 9. Circulating inflammatory cytokines in patents and controls

	Patients (n=361)	**Controls** (n=375)	**p-value**
IL-1α (pg/mL)	0.00 (0.00-0.91)	0.00 (0.00-0.91)	ns
IL-1β (pg/mL)	0.05 (0.00-0.81)	0.00 (0.00-0.26)	<0.05
IL-2 (pg/mL)	5.68 (0.00-7.09)	1.51 (0.00-6.17)	<0.0001
IL-4 (pg/mL)	0.00 (0.00-1.49)	0.00 (0.00-1.50)	ns
IL-6 (pg/mL)	1.31 (0.65-2.75)	0.59 (0.16-1.84)	<0.0001
IL-8 (pg/mL)	3.72 (2.27-5.75)	1.76 (0.88-3.43)	<0.0001
IL-10 (pg/mL)	0.00 (0.00-0.39)	0.00 (0.00-0.44)	ns
IFN-γ (pg/mL)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	ns
TNF-α (pg/mL)	3.92 (3.23-4.81)	3.48 (2.86-4.23)	<0.0001
MCP-1 (pg/mL)	160.2 (131.9-199.5)	159.2 (127.8-198.2)	ns

Values are median (interquartile range). Significance level in Mann-Whitney U test.

Table 10: Spearman rank correlations between MMP-3 and MMP-1 concentrations and inflammatory cytokines

	MMP-3			MMP-1		
	P+C	**Patients**	**Controls**	**P+C**	**Patients**	**Controls**
IL-1α	-0.01	0.03	-0.06	**0.09***	**0.15***	0.03
IL-1β	-0.03	-0.05	0.03	**0.10***	0.06	**0.14***
IL-2	-0.06	-0.03	-0.02	**-0.10***	**-0.16***	-0.04
IL-4	0.00	0.03	-0.03	**0.20***	**0.23***	**0.17***
IL-6	**-0.10***	-0.07	-0.01	**0.16***	0.09	**0.23***
IL-8	**-0.07***	-0.04	0.03	-0.02	**-0.15***	**0.11***
IL-10	0.03	0.06	-0.01	**0.27***	**0.24***	**0.30***
IFN-γ	-0.04	0.02	-0.08	-0.01	-0.00	-0.02
TNF-α	0.02	0.05	0.07	**0.14***	0.03	**0.23***
MCP-1	0.05	0.06	0.05	**0.09***	**0.14***	0.03

* $p<0.05$, figures in bold, P+C=patients and controls

GENERAL DISCUSSION

The focus of this thesis is the evaluation of the roles of MMP-3 and MMP-1 in coronary artery disease and myocardial infarction, and their potential modulation by inflammation. This was performed by genetic analyses of known and novel polymorphisms in the MMP-3 and MMP-1 genes, biochemical analyses of circulating levels of MMP-3, MMP-1 and various cytokines, and angiographic analyses of the extent and severity of CAD.

MMP-3 genotype-phenotype relation

Several case-control studies have been conducted to evaluate the association between the MMP-3 -1612 5A/6A promoter polymorphism and the risk of suffering MI (Table 3). Five studies out of eight concluded that the 5A-allele is associated with a higher risk of MI[80,96-99], whereas one study, by far the largest one, indicated that the 6A-allele is associated with a higher risk[100]. In two studies there was no difference according to genotype[88,95]. In the study reported in paper I, we genotyped a well-defined case-control cohort comprising 387 post-infarction patients and 387 control subjects (SCARF) for the MMP-3 -1612 5A/6A promoter polymorphism. The allele frequency did not differ significantly between the two groups, yielding no excess risk of MI for either allele. Importantly, our study was not designed to detect differences in allele frequencies between the groups, since it was too small to detect minor differences in risk of MI. Furthermore, we did not detect any difference in the extent or severity of CAD between genotype groups. In the literature there are ten studies addressing this latter question, or progression of CAD using repeated angiographies (Table 2). Of these studies, eight showed that the 6A-allele is associated with more severe disease, as evaluated by angiography[79,80,84,88-92], whereas one proposed the 5A-allele[94]. However, the largest study detected no differences[93]. Thus, our study could not confirm the notion that the 5A-allele increases the risk of MI whereas that the 6A-allele promotes progression of atherosclerosis[150,151]. Interestingly, when reviewing the published studies on these issues, the largest ones on risk of MI and on CAD severity, respectively, presented the deviating results. In addition, in a large family-based case-control study, no association between MMP-3 -1612 5A/6A genotype and MI or CAD could be detected[108]. In fact, the problem of underpowered genotype-phenotype association studies is widespread, and one cannot exclude a publication bias where smaller studies presenting a genotype-specific effect are reported whereas those that failed to demonstrate an effect may not have been published. To finally assess the possible association of the MMP-3 -1612 5A/6A polymorphism with MI and CAD, we need larger, well-conducted longitudinal cohort studies or prospective population-based studies with refined definitions of the phenotype.

Functional *in vitro* studies on the MMP-3 -1612 5A/6A promoter polymorphism have shown the 5A-allele to be associated with increased expression of MMP-3[48,81]. Against this background, we expected the serum MMP-3 concentrations to be higher in individuals carrying the 5A-allele. In contrast, our results showed a very clear increase in serum MMP-3 concentration with increasing numbers of 6A-alleles (paper I). In a study of patients with rheumatoid arthritis, Mattey et al. had the same paradoxical finding[83]. In order to further elucidate this issue, we performed genotype analysis in the TREOC study as reported in paper II. This second study replicated our results in SCARF. In addition, White and co-workers presented similar results obtained in CAD patients at the American Heart Association Scientific Sessions 2005[84]. To the best of my knowledge, no one has reported homozygotes for the 5A-allele to have higher levels of circulating MMP-3 than heterozygotes and/or 6A homozygotes, whereas there are three studies reporting no difference between genotypes[85-87]. These conflicting findings between *in vitro* and *in vivo* studies are difficult to explain, but the results are very clear and convincing, as they have been reproduced in five different

study groups, counting the patient and control groups of the SCARF cohort as two independent samples. Importantly, the serum MMP-3 concentration appears to be related to MMP-3 gene expression and not to altered clearance of the protein since the serum concentrations are associated with the promoter polymorphism.

It may be argued that the circulating levels have little to do with what is occurring within the vessel wall as MMPs may bind to connective tissue and be retained within the ECM[152]. However, it would be expected that leakage of protein into the blood stream would be correlated with expression of the protein. It cannot be excluded that there are other sources of circulating MMP-3 than the vessel wall. Also, the impact of the promoter polymorphism may be different during inflammatory conditions, which are present in the atherosclerotic vessel wall. The functional experiment of Ye et al. was performed *in vitro,* and the cells may have altered their behaviour when extracted from their original environment[48]. However, the experiment of Medley et al. where MMP-3 mRNA levels were higher in the 5A homozygotes, as measured in skin biopsies, argues against this explanation[81]. This result was replicated in aortic samples from a different, though rather small (n=7), study group to confirm that cell type does not influence the expression.

Circulating MMP-3 and MMP-1

Several MMPs have been indicated to be expressed in atherosclerotic tissue but not in non-affected vessels, suggesting an essential role in atherosclerosis[38-40]. Efforts to reinforce this conclusion by analysing circulating levels of MMPs in case-control studies have not reached consistent results, and most of the studies included less than 100 individuals[55-59,61,153]. We investigated MMP-3 and MMP-1 in the SCARF case-control study consisting of a total of 774 individuals. The serum MMP-3 concentration was found to be lower in the patient group as compared to the control group (paper I), whereas the plasma MMP-1 concentrations did not differ between the groups (paper IV). Importantly, the blood samples for these analyses were drawn three months after the acute event when the acute inflammation due to myocardial damage had subsided. The results therefore could be regarded as reflecting the resting inflammatory state of the patient. A problem inherent in this approach is the potential influence of the medication that the patients, but obviously not the control subjects, received during the three months preceding blood sampling. Experimental studies have for example suggested that statins reduce the expression of MMP-1[154], MMP-2[155], MMP-3[156] and MMP-9[157-160]. Only 35% of the patients in SCARF were on lipid-lowering therapy as this study was conducted before the widespread use of statins in secondary prevention. However, the levels of neither MMP-3 nor MMP-1 differed between patients on statins and those who had not received this therapy. In addition, excluding patients on statins from the case-control analysis of MMP-3 and MMP-1 did not change the initial results reported in papers I and IV, nor did ACE-inhibitors influence the results. Similar analyses of aspirin and betablockers are hazardous as the vast majority of the patients were constantly on these medications, but no difference between patients with and without these medications were detected.

As MMPs are indicated to be involved in plaque rupture, it is plausible that the MMP-3 and MMP-1 concentrations might have been higher in the acute stage of MI. To evaluate this issue, in the case of MMP-3, we measured the serum MMP-3 concentration in the TREOC cohort of STEMI patients where we had blood samples available both from the acute stage and the three months follow-up visit. The analysis revealed that the MMP-3 concentration is actually lower in the acute stage, 0 and 48 hours after admission, than in the recovery period, three months after the index event (paper II). This is in contrast to two earlier reports published on the levels of MMP-3 in the acute conditions of CAD as both claimed higher concentrations of MMP-3 in ACS than in stable CAD patients and also in comparison with controls[56,57]. In both these studies, there were 20 individuals, or less, included in each study group. Also, in one of the studies, the difference was only seen in coronary sinus blood whereas no difference was detected in blood drawn from the aortic root[56]. In addition, none of the study groups were followed over time. Another study of stable angina pectoris demonstrated higher levels in patients than in controls[58]. In contrast, other studies of stable CAD or risk factor conditions such as hyperlipidemia

and type 2 diabetes, showed lower concentrations of MMP-3 in affected subjects[55,59,60]. These diverging study results may of course indicate that the circulating levels of MMP-3 are not related to acute MI or other manifestations of CAD, but histopathological studies of atherosclerotic plaques have clearly indicated MMP-3 to be present in affected but not in normal vessels, indicating a role of MMP-3 in the pathogenesis of atherosclerosis[38,39]. Furthermore, most of these studies are quite small in terms of numbers of participants. In fact, both our studies, detecting lower MMP-3 concentrations in MI patients and during the acute stage of MI, are larger than the previously performed.

Clearance of MMPs is of course a process that can influence the circulating levels of MMP-3 and MMP-1. MMP clearance is quite unexplored, and little is known about the mechanisms. The low-density lipoprotein receptor-related protein (LRP) is proposed to contribute to clearance of at least some of the MMPs[161]. In this context it is important to note that the circulating MMP-3 concentration is clearly influenced by the MMP-3 -1612 5A/6A promoter polymorphism suggesting that MMP-3 levels reflect the transcriptional activity rather than degradation or clearance processes.

On the assumption that circulating levels of MMPs reflect the amount of MMPs in the vessel wall, the finding of lower MMP-3 concentration in the acute stage of MI as well as in postinfarction patients compared with control subjects speaks against elevated MMP-3 concentrations in atherosclerotic tissue being an important feature of fibrous cap weakening and plaque rupture. This uncertainty is strengthened by recent studies in MMP-3 deficient mouse models. Two studies of apoE/MMP-3 double knockout mice have demonstrated larger cross-sectional atherosclerotic plaque areas[43, 44], with a lower content of SMCs and an increased number of buried fibrous layers, features of a less stable plaque[43].

Studies on circulating MMP-1 concentrations in relation to MI and stable CAD seem to be as contradictory as the studies on MMP-3. In two studies, MMP-1 has actually been undetectable[65,66], while other studies have shown either higher MMP-1 concentrations[56,72], notably only in ACS patients, or no difference in case-control studies[64,68,70,74]. Several studies have detected a transient increase in the MMP-1 concentration during the initial phase of MI, but MMP-1 appears to return to baseline concentration already within the first month[69,71,73]. Our result of no difference in plasma MMP-1 concentration between post-infarction patients and controls at three months after the index event is in agreement with these previous studies (paper IV).

Coronary angiography and MMPs

Coronary angiography is still the routine method to detect hemodynamically significant stenoses in the coronary circulation. In the future, it is likely to be replaced by multi-slice computer-assisted tomography (CT) or magnetic resonance imaging (MRI), at least for the purpose of ruling out significant CAD[162]. Today it is widely recommended to perform coronary angiography followed by appropriate intervention when needed, in patients presenting with ACS, and also in most instances of stable CAD[163]. In most of the studies discussed above, routine coronary angiography was performed, but no correlation was found between number of diseased vessels and MMP-3 concentration[56,58]. Similar results were noted for MMP-1[56,64].

The only angiographic observation reported to correlate with MMP-3 concentration was signs of ectasia or aneurysm. Finkelstein et al. reported higher MMP-3 concentrations in patients with only focal signs of coronary ectasia whereas patients with generalized ectasia had lower concentrations[164]. In contrast, Tengiz et al. detected higher MMP-3 concentrations in patients with CAD and coronary aneurysm as compared with patients with CAD but no coronary aneurysm[153]. The pathophysiology of aneurysm formation in coronary arteries is probably slightly different from that of the obstructive changes characteristic of classic CAD, even though atherosclerosis is apparent also in aneurysmal disease. In this context, our finding of increasing levels of MMP-3 with increasing numbers of diseased major coronary arteries in paper II is very interesting as this is the first study to report a possible correlation between the circulating MMP-3 concentration and extent of CAD. On the other hand, the results in the SCARF study (paper I) were not entirely clear as there were increasing MMP-3 concentrations from 0- to 2-vessel

disease whereas patients with 3-vessel disease had again lower concentrations, which did not differ significantly from the levels of patients with 0-vessel disease. Like others, we did not detect any correlation of MMP-1 levels with angiographic measurements (paper IV).

The studies on the MMP-3 -1612 5A/6A promoter polymorphism have generally claimed that the extent of CAD is higher in carriers of the 6A-allele, assuming a relation to lower levels of MMP-3, consistent with the gene expression study of Ye et al.[48], but the circulating MMP-3 was actually not measured. In our studies we have shown that the serum MMP-3 concentration is higher in carriers of the 6A-allele, thus suggesting that higher MMP-3 concentrations are associated with more extensive CAD. Contradictory to this, however, is the finding of an inverse correlation between MMP-3 concentration and plaque area in women (paper I), but as will be discussed, there are several gender differences in this field. It should also be stressed that coronary angiography is not the best method to estimate the true plaque area as it is only the area of the plaque that is protruding into the lumen that is measured. IVUS is in most respects a superior method to measure true plaque area, but at the time when the SCARF study was conceived and conducted this method was not available in our institution.

Gender differences in MMP-3 and MMP-1

Both MMP-3 and MMP-1 levels were found to be lower in women than in men; for MMP-3 concentration there was a striking, almost two-fold, difference (papers I and II). Gender differences in atherosclerosis and CAD are well-known since decades, but the underlying causes are not fully understood. These differences may be mediated by the sex steroid hormone receptors, which when activated, act as transcription factors, modulating gene expression through binding to DNA response elements. There are many indications that oestrogen plays a protective role in pre-menopausal women, but the belief in hormone replacement therapy as the rescuer to all women came to nought when tested in prospective randomised trials[118,119]. This was disappointing and surprising as much of the molecular and cellular knowledge about the effects of oestrogen pointed towards a benefit of oestrogen substitution[117,165].

In a study of cultured human aortic SMCs, testosterone was found to increase both transcription and translation of the MMP-3 gene, rendering a two-fold increase in protein levels as compared to cells cultured with the combination of 17 β-oestradiol and progesterone[131]. In addition, cells cultured with the female sex hormones produced an elastin/collagen ratio that was essentially higher than in cells cultured with testosterone. In another study of cultured osteoarthritic chondrocytes, the secretion of MMP-1 was significantly reduced by 17β-oestradiol, also when stimulated by a low concentration of TNF-α, but not IL-1β, whereas in this study there was no effect on MMP-3 secretion[130]. Whether these mechanisms demonstrated *in vitro* are responsible for the observed gender differences needs to be proven.

The sex difference in circulating MMP-3 levels revealed in the SCARF study (paper I) corresponded in terms of magnitude with the *in vitro* studies, whereas the difference in TREOC was less pronounced but still significant (paper II). The gender difference in circulating levels of MMP-3 was first reported by Manicourt et al. investigating osteoarthritis and healthy controls who described a two-fold higher serum concentration of MMP-3 in males as compared to females[166]. In that study, the serum MMP-3 concentration increased with age, a correlation that we could not confirm in the SCARF cohort, but which was evident in the TREOC cohort. These diverging results may possibly be due to the upper age limit of 60 years in the SCARF cohort. Increased MMP-3 concentration in female healthy controls has also been reported by Zucker et al.[49], whereas Beaudeux et al. did not detect any gender difference in hyperlipidemic, otherwise healthy subjects[61]. In contrast to our finding in paper IV, Manicourt et al. did not detect any gender difference in serum MMP-1 level. Even though both sexes are included in the majority of the studies exploring circulating levels of MMPs, very few report the results according to gender.

MMP-1 haplotype effect

Association studies of candidate genes and their polymorphisms in atherosclerotic diseases have up to recently focused on single polymorphisms. Since these conditions are really complex and multi-

factorial, this approach seems to be unlikely to succeed in determining genetic susceptibility[76]. Haplotype analysis of interrelated polymorphisms within a single gene or in closely related genes has emerged as a refined method to explore genetic influence on disease processes. Haplotype analysis may reveal associations that are not obvious when analysing the different polymorphisms individually[167-169]. Our study of the MMP-1 gene polymorphisms (paper III) constitutes a good example as none of the seven identified promoter polymorphisms were individually associated with increased risk of MI whereas the combinations of two of the polymorphisms, -519 A/G and -340 T/C, were associated with the risk of MI. In our study, the -1607 G/GG polymorphism did not appear as a predictor of the risk of MI, neither in isolation, nor in the haplotype analysis. This might be explained by the close linkage between the -1607 G/GG and -519 A/G polymorphisms[114].

The fact that haplotype analyses was superior to individual SNP genotype analyses as regards prediction of risk in our study could possibly be due to the complex interactions between the promoter region and the transcription complex. The different polymorphisms do not act independently, but rather interact in modifying binding sites for transcription factors. In addition, different polymorphisms may not be simply additive but rather modulate each other's binding properties as proposed by Terry et al. in a study of haplotype effects in IL-6 gene transcription[170]. Another explanation could be that haplotypes are more likely to show linkage disequilibrium with an as yet unknown causal gene variant[171].

Functional studies of the haplotypes proposed in paper III to be associated with MI supported the statistical finding of a divergent risk of MI. As indicated by EMSAs, both the -519 A/G and -340 T/C polymorphisms seem to influence the capacity of binding nuclear proteins to the promoter region. Efforts to identify the nuclear proteins involved in the DNA-protein complexes were unsuccessful, indicating that the transcription factors in question are probably not present in the current databases. Nevertheless, this finding suggests the -519 A/G and -340 T/C polymorphisms role of alter the transcription of the MMP-1 gene. This suggestion was strengthened by the transient transfection studies where the firefly luciferase reporter gene was less expressed by the A_{-519}-C_{-340} and G_{-519}-T_{-340} promoter haplotypes than by the A_{-519}-T_{-340} promoter haplotype. In addition, real-time RT-PCR of atherosclerotic carotid plaques revealed lower levels of MMP-1 mRNA in plaques from patients with the A_{-519}-C_{-340} and G_{-519}-T_{-340} haplotypes than in plaques from patients with the A_{-519}-T_{-340} haplotype. No plaque from a carrier of the G_{-519}-C_{-340} haplotype was eligible. These experimental studies indicate a decreased MMP-1 expression in lower risk haplotypes, coherent with the hypothesis of increased collagenolysis in the vulnerable atherosclerotic plaque contributing to plaque rupture[40].

Inflammation and MMPs

In the past decades, evidence has accumulated that inflammation is an important feature of atherosclerosis, both in the initiation phase and in the advanced lesions where the complications of atherothrombosis occur[4,5,172]. Inflammation has a fundamental purpose in protecting the organism from microbial and viral attacks and also plays a key role in wound healing. Without inflammation we would have no defence against infectious diseases. However, the inflammatory process can also be harmful. It has long been evident that even a low grade of chronic inflammation could injure the tissues, as is the case in well-known inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease. Less obvious is the harmful inflammation of the intima of the vascular wall in atherosclerosis. In the initiation of atherosclerotic plaque formation, modified lipoproteins, among other antigens, are thought to initiate an inflammatory response. The modified, predominantly oxidized, LDL-particles activate the endothelial cells, which in turn attract blood-derived inflammatory cells into the intima.

As long as the phagocyting macrophages keep up with the modified lipoproteins, the inflammation is advantageous, but when the lipid burden is growing, the inflammation aggravates and the macrophages get overloaded with lipids and become apoptotic. The formation of an atherosclerotic plaque begins and the inflammatory process is maintained. Activated inflammatory cells secrete cytokines that further aggravate the inflammation and maintain the vicious circle. The

inflammatory cytokines are suggested to promote the weakening of the plaque's fibrous cap partly by reducing the proliferation of SMCs and their secretion of procollagen, partly by enhancing the production and activation of MMPs that are mainly produced by macrophages[9]. SMCs are inhibited by IFN-γ and IL-1, and TNF-α is known to up-regulate the transcription of MMPs, whereas TGF-β decreases MMP transcription[9,33,34]. However, the interaction between inflammatory cytokines and MMPs is intricate and not yet fully understood.

In paper IV we investigated the inter-relationship between a panel of inflammatory cytokines and the proteases MMP-3 and MMP-1 in the SCARF case-control study. All measurements were performed in plasma or serum samples derived from venous blood, i.e. reflecting the circulating levels of cytokines and MMPs. The plasma levels of IL-2, IL-6, IL-8 and TNF-α were significantly elevated in the postinfarction patients. In addition, there was a trend towards more patients with detectable levels of IL-1β than controls, and the detectable measurements were significantly higher in patients than in controls. Also, as reported in paper I, patients had higher hsCRP levels than controls. These results indicate that a more active inflammatory state prevails in patients than in controls, as we presume that the inflammation caused by the myocardial damage itself had subsided at the time of blood sampling, three months after the MI. These results extend those of several previous smaller studies of separate cytokines in relation to MI and CAD, as the SCARF cohort is larger and all cytokines were analysed simultaneously[19,20,22]. Also, the lack of difference in IL-10 concentration between patients and controls is in accordance with two earlier case-control studies[20,23], whereas another study has shown higher IL-10 levels in CAD patients[19].

The most interesting finding in paper IV is the difference between MMP-3 and MMP-1 levels as regards their respective correlations with several inflammatory cytokines. The corollary is that the expression of MMP-3 seems to be less influenced by inflammation than the expression of MMP-1. Recently, studies of different MMP-deficient apoE-null mice have indicated divergent effects of different MMPs in atherosclerosis[43]. In particular MMP-3 was suggested to have atheroprotective properties. The ApoE/MMP-3 double knockout mice showed larger plaque area, fewer SMCs and more buried fibrous layers, suggesting previous plaque rupture, but there was no difference in macrophage content of the intima as compared with apoE single knockout mice[43]. This might be interpreted that the atherosclerotic plaques have a more vulnerable profile in the absence of MMP-3. On the other hand, Silence et al. reported a lower content of macrophages in the atherosclerotic lesions of the apoE/MMP-3 double knockout mice than in the apoE single knockout mice, indicating less inflammation in the plaques of the double knockout mice[44].

In the pioneering work of Henney et al., MMP-3 gene expression was detected in atherosclerotic plaques, both in macrophages and SMCs. However, the plaque that showed the most extensive MMP-3 expression was lacking a lipid core[39] and presumably less inflamed. In addition, the *in vitro* studies of Whatling et al. demonstrated that activated macrophages increase their expression of the MMP-1, MMP-2, MMP-9 and MMP-14 genes but, notably, not of MMP-3[37]. Also, in a study of carotid artery plaques, classified into four groups according to histological features, the MMP-1 transcript levels were nearly eight-fold higher in plaques with a thin cap as compared with plaques featuring a thick cap, whereas there was only a slight, and not statistically significant, increase between those plaque groups in MMP-3 transcript levels[63].

Adhesion molecules are important in the initiation of plaque formation as they promote adhesion and attraction of inflammatory cells to the site of activated endothelium. In a recent case-control study of type 2 diabetes, there was an inverse correlation between the concentrations of circulating MMP-3 and soluble intercellular adhesion molecule (sICAM-1) in diabetic subjects[60]. In addition, the diabetic subjects had significantly lower concentrations of MMP-3 than the control subjects[60], which is interesting as diabetic patients are known to run an increased risk of cardiovascular events. Devaraj et al. investigated whether a pro-inflammatory phenotype could be identified by hsCRP, comparing a number of proposed pro-

inflammatory molecules between a group of healthy individuals with high hsCRP (median level 3.7 mg/L) and a group of healthy individuals with low hsCRP (median level 0.21 mg/L). Levels of MMP-3 and MMP-9 were investigated, and the two groups did not differ in MMP-3 concentration, whereas the MMP-9 concentration was higher in the high hsCRP group[173]. These recent studies support the proposed hypothesis of a different regulation of MMP-3 that is less influenced by inflammation.

The most consistent evidence of inflammatory regulation and involvement in plaque rupture exists for MMP-9. Circulating plasma levels of MMP-9 predicted adverse outcome in a study of CAD patients[174]. In addition, MMP-9 gene expression was 10.8-fold higher in human macrophages than in any other of 55 studied human tissues, using DNA microarrays[175]. No other MMP genes were among the 23 genes identified to have increased expression in macrophages. In a study of carotid artery plaques excised from patients undergoing carotid endarterectomy, the plaque extract concentrations of IL-6 and IL-8 were positively correlated with those of MMP-2 and MMP-9[176]. Also, the concentrations of IL-6, IL-8, MMP-2 and MMP-9 were all higher in unstable carotid plaques as compared with stable ones.

Ever since the work of Henney et al. and Galis et al. in the mid 90's[38,39], which demonstrated that several MMPs are present in atherosclerotic tissues, the prevailing opinion has been that all MMPs promote progression of atherosclerosis and, at least partly, are the cause of plaque rupture by degrading the fibrous cap. The notion of a uniform, destructive role of MMPs in atherosclerosis is now being questioned, and our results speak in favour of reconsideration. Although preclinical studies of MMP inhibitors have been promising in terms of reducing remodelling and intimal hyperplasia, the clinical trials conducted so far have been disappointing as they could not reproduce the preclinical findings[177]. Also, broad-spectrum MMP inhibitors have been tested in clinical trials in the cancer field, but failed success because of severe side effects due to interference with normal tissue function. The disappointing results of these clinical studies reinforce the hypothesis of differential effects of individual MMPs and emphasize the need for more detailed knowledge about the role of individual MMPs in atherosclerosis.

CONCLUSIONS

- The circulating level of MMP-3 is markedly influenced by the MMP-3 -1612 5A/6A promoter polymorphism.
- Reduced MMP-3 concentration seems to be associated with MI, whereas increased concentration may promote progression of stable CAD.
- The MMP-3 concentration is clearly influenced by gender, being roughly two-fold higher in men than in women. Similarly, the MMP-1 concentration seems to be lower in women.
- MMP-1 promoter polymorphisms influence the risk of MI.
- Inflammation is suggested to exert a stronger influence on the MMP-1 concentration than on the MMP-3 concentration.
- The conclusions of this thesis reinforce the novel alternative hypothesis that different MMPs have divergent effects on atherosclerosis and CAD.

FUTURE PERSPECTIVES

- The roles of individual MMPs in atherosclerosis and CAD need to be investigated further and better defined.
- The potential use of plasma concentrations of individual MMPs as predictors of CAD and MI needs to be further evaluated in large prospective studies.
- Future studies of MMPs in CAD will require thorough definitions of different etiologies of MI, presence of coronary stenosis, and stable versus vulnerable plaques.

ACKNOWLEDGEMENTS

The work reported in this thesis has been conducted at Karolinska Institutet, partly at the Cardiology Unit, Department of Clinical Science, Danderyd Hospital (DS), partly at the Atherosclerosis Research Unit, King Gustaf V Research Institute (GV), Department of Medicine, Solna. Here I want express my sincere gratitude to all persons without whom this thesis never have been completed, in particular I wish to thank:

My three supervisors:

Associate professor **Carl-Göran Ericsson**, for encouraging me to go into research and never losing faith in me, for good advice and sharing wisdom, and as the Head of the Department of Medicine, Danderyd University Hospital, for being such a good model for empathic leadership.

Associate professor **Per Eriksson**, for introducing me to the exciting field of proteases and molecular genetics, teaching me to spell with only four letters, and for challenging intellectual discussions and endless patience with my thousands of questions.

Professor **Anders Hamsten**, Head of the Atherosclerosis Research Unit, Department of Medicine, Solna, for your great knowledge in atherosclerosis and sharing it with me, for providing excellent research facilities, and for beautiful and sophisticated linguistic refinement of the manuscripts and this thesis.

Dr **Lott Bergstrand**, PhD, co-author and former supervisor, for introducing me to, and teaching me all I know about coronary angiography.

Associate professor **Angela Silveira**, co-author, for teaching me all about ELISA and for your kind and prompt feedback on the manuscripts.

Karin Malmqvist, Head of the Cardiology Unit, Department of Medicine, Danderyd University Hospital, for providing time and space for science, for being so immensely enthusiastic and encouraging, and for promoting such a pleasant working atmosphere in our clinic.

Hans Persson, former Head of the Cardiology Unit, Department of Medicine, Danderyd University Hospital, for invaluable support during hard times in both life and science.

Pia Lundman, co-author, close friend and room-mate for ages, for helping with patient inclusion and management, uncountable hours on the phone about scientific, clinical and personal matters, and for always spurring me on to new conquests.

My co-authors **Jacob Odeberg**, Royal Institute of Technology, **Per Tornvall**, Department of Cardiology, Karolinska University Hospital, **Johannes Hulthe** and **William McPheat**, Molecular Pharmacology, AstraZeneca, Mölndal, for valuable contributions to the manuscripts.

My co-authors **Eve Pearce**, **Angharad R. Morgan**, **Charles Cox**, and **Shu Ye** at Human Genetics Division, School of Medicine, University of Southampton, Southampton, United Kingdom, and **David-Alexandre Tregouet**, INSERM U525, Faculté de Médecine, Hôpital Pitié-Salpêtrière, Paris, France for fruitful collaboration resulting in paper III.

Susanna Boquist, co-author, for recruitment of patients included at the Karolinska University Hospital Solna and the control subjects.

Lars-Erik Strandberg, Head of the Department of Internal Medicine, Norrtälje Hospital, for patient recruitment and compilation of the clinical database in the TREOC study. **Göran Svensson** and his colleagues at Norrtälje hospital for generously providing the SCARF study with patients.

Ann-Marie Ekman for taking so good care of the SCARF patients and, not least, of their blood samples, **Marie-Louise O'Konor** for working in the dark, reviewing so many angiographies and taking care of the patients in the TREOC study, and **all former and present staff** at the "Heart Lab", Danderyd University Hospital for excellent work and kind helpfulness, and for keeping order in the fridges.

Barbro Burt for patient hands-on tuition on PCR and pyrosequencing, **Karin Danell-Toverud** for guarding databases and fridges, and **Anita Larsson**, **Karin Husman**, and **Birgitta Söderholm** for excellent technical assistance.

All former and present staff at the Centre for Clinical Cardiovascular Research, Karolinska University Hospital Solna, for taking care of patients and voluntary controls.

Alejandro Bertorello, **Johan Björkegren**, **Ewa Ehrenborg**, **Rachel Fisher** and **Ferdinand van't Hooft**, senior researchers, and all **present and former PhD-students, post-docs and friends**, at GV for contributing thoughts, interesting discussions on molecular biology and for providing an exciting academic environment, and the rest of the **staff** for taking care of all small but important matters. Special thanks to **Per Sjögren** for holding the "Molecular Book Club" together and to **Josefin Skogsberg** for joyful enthusiasm on every topic in life and science.

Håkan Wallén for encouragement and support of my work, and **Peter Henriksson**, **Magnus Edner**, and **Thomas Kahan**, senior researchers at the Cardiology Unit, Department of Clinical Science, Danderyd University Hospital, for taking interest in the progress of my thesis.

Marie Björnstedt-Bennermo, close friend and colleague, for nice chats on science and life, and for priceless advice on how to survive the finishing of thesis writing.

Cathrine Edström-Plüss, great friend and colleague, for taking care of clinical duties during my leave and for taking so much interest in my well-being.

Toomas Särev, for bringing new technologies to the cath lab and relieving me of administrative duties, and all the **doctors and nurses** at cath lab for perfoming all the excellent angiographies of the SCARF and TREOC studies, and for contributing to such a friendly and informal work setting.

All former and present friends, **colleagues and staff**, at the Department of Medicine, Danderyd University Hospital, for keeping up a friendly and creative atmosphere.

Nina Rehnqvist, Executive Director of SBU – The Swedish Council on Technology Assessment in Health Care, for igniting the research spark and keeping it from going out during the initial years of my work.

Elisabeth Berg for making statistics understandable and for sensible advice.

Karin Björklund-Jonsson for inestimable help in transforming my bleak Word documents into this elegant publication.

Magnus Mossfeldt for indispensable and knowledgeable IT-support.

Hans Samnegård, my uncle, for offering me my very first exciting experience of medical research and for being such a good role model for our profession.

Ingegerd and **Gunnar**, my beloved parents, for endless love, care and support in whatever I have decided to do in life.

Eva, my sister, and **Arne** for generously providing Lady Väs and Skälsmara whenever I needed recreation, **Dag,** my brother, **Susanna**, **Johanna** and **Pontus**, for warm hospitality and a constantly open house, and **Karin,** my sister, **Stefan**, **Rasmus** and **Leo**, for always being so wise, positive, fun and encouraging.

All my **friends** and **neighbours**, to whom I have been invisible lately, for caring, support and encouragement.

And to all the patients, patiently undergoing interviews, blood sampling and angiography, without you there would be no clinical research!

Thank you!

This work was supported by grants from the Swedish Heart-Lung foundation, Karolinska Institutet and the Stockholm County Council. In addition, the separate studies were supported by grants from the Swedish Medical Research Council, AFA insurance, the Torsten and Ragnar Söderberg foundation, the King Gustaf V and Queen Victoria foundation, the King Gustaf V 80th birthday foundation, the Serafimer foundation, the Petrus and Augusta Hedlund Foundation, the British Heart Foundation, the European Union Framework Programme 6 Degradome project and AstraZeneca.

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